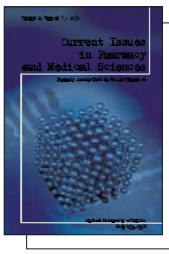

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Quantitative analysis of biofilm formation by oropharyngeal *Candida albicans* isolates under static conditions by confocal scanning laser microscopy

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ABSTRACT

Candida albicans may colonize natural or artificial surfaces, leading to formation of the biofilm. Infections associated with the biofilm formation are important therapeutic problem. In this paper, we present data concerning the biofilm formation under static conditions by oropharyngeal isolates of *C. albicans* on a glass surface using confocal scanning laser microscopy (CSLM). The areal parameters describing the architecture of biofilm and its development, *i.e.* the areal porosity, the length of edge line, the length of skeleton line, were calculated. The changes in values of these parameters during the biofilm formation by *C. albicans* were similar for biofilm consisting of only blastospores as well as the biofilm consisting of blastospores and filamentous elements (hyphae or/and pseudohyphae). However, the thickness of *C. albicans* biofilm consisting of blastospores and filamentous elements was much higher than that consisting of only blastospores. The heterogeneity may be regarded as an important feature of the yeast biofilm including *C. albicans*.

Keywords: *Candida albicans*, biofilm, confocal scanning laser microscopy

INTRODUCTION

Microbiota biofilms occur and persist in both the environment and in the human body; in the latter case especially in connection with medical devices. The process by which microorganisms colonize artificial (*e.g.* glass, polychloride vinyl, polystyrene, silicone) or natural surfaces (*e.g.* epithelial cells) involves a series of steps starting with physicochemical interaction between microbial cells and substratum, followed by cell adhesion, multiplication and differentiation, leading to formation of the mature biofilm [7,12,14,16]. Yeasts are found in a variety of natural habitats. Some of them, *e.g. Candida* spp., being a component of normal microflora colonizes skin and mucous membranes of mouth, upper respiratory tract, intestinal tract or vagina; the predominant species is *Candida albicans*. These microorganisms can be regarded as an important opportunistic pathogens involved in infections associated with the biofilm formation on mucous membranes or indwelling medical devices being an *in vivo* substrata for the yeast cell adhesion [2,5,9]. A wide range of biomaterials used in clinical practice have been shown to support colonization and biofilm formation by *Candida* spp. [14,18].

Several microscopic techniques have been developed in order to understand the biofilm structure and its physiology *in situ*, *e.g.* non-invasive confocal scanning laser microscopy – CSLM [13,15,20,22]. In this paper, we present data concerning the biofilm formation at various phases of its development *in vitro* by oropharyngeal isolates of *C. albicans* on a glass surface under static conditions, using CSLM. This technique allowed to calculate the areal parameters describing the architecture of *C. albicans* biofilm during its formation and maturation.

MATERIALS AND METHODS

Yeast strains and culture conditions. *Candida albicans* isolates were obtained from oropharynx of patients with lung cancer undergoing pulmonary resection. The yeast suspensions were stored at -20°C in 50% glycerol and then cultured on Sabouraud dextrose agar at 30°C for 48 h; before each experiment, the isolates were subcul-

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tured on Sabouraud glucose broth (further called Sabouraud medium) at 30°C for 48 h.

Confocal scaning laser microscopy (CSLM) analysis. The standardized yeast suspensions (optical density of 0.5 McFarland standard, *i.e.* 5 x 10^6 colony forming units mL^{-1}) in Sabouraud medium were prepared. In order to assay adhesion process, 350 µL of inoculum was added to four of the eight-well polystyrene culture chamber and then incubated for 1 h at 35°C, followed by gentle washing of the wells with sterile phosphate-buffered saline (PBS) to remove nonadherent cells. Next, to each well 200 µL of solution containing 0.1 mg mL^{-1} of concanavalin A Alexa Fluor 488 conjugate (CAAF) was added and incubation was continued for 45 minutes at 35°C. In order to assay biofilm formation at early stage, 350 µL of inoculum was added to four of the eight-well culture chamber and then incubated for 24 h at 35°C, followed by gentle washing of the wells with sterile PBS to remove nonadherent cells. Next, to each well 200 µL of solution containing CAAF was added and the incubation was continued for 45 minutes at 35°C. In order to assay the mature biofilm, 350 µL of inoculum was added to four of the eight-well culture chamber and then incubated for 24 h at 35°C. Next, nonadherent cells were removed by careful well rinsing with sterile PBS and then fresh Sabouraud medium was added. Medium changing and the culture chambers washing procedures after overnight incubation at 35°C were repeated twice (a total incubation period lasted 72 h). After this time 200 µL of solution containing CAAF was added and incubation was continued for 45 min at 35°C. All assays were carried out in four replicates. The pictures for planimetric measurements were carried out in two-dimensional scans at a magnification of x 50. The planimetric analysis was perfomed using Image J. v. l. 36b, Wayne Rasband, National Institutes of Health, USA. The statistical analysis was performed by applying Shapiro-Wilk's and Levene's tests using Statistica 6.0. All tests were performed with a confidence level of 95%.

RESULTS

Three parameters describing biofilm formation by oropharyngeal *C. albicans* isolates - the areal porosity, the length of edge line, the length of skeleton line, were calculated using CSLM technique at various phases of biofilm development, *i.e.* after 1 h incubation (adhesion step), 24 h incubation (the early stage of biofilm formation) and 72 h incubation (the mature biofilm); the overall incubation period lasted 72 h (Tab. 1).

These parameters showed a similar tendency during biofilm formation by two isolates of *C. albicans*. The field of culture chamber occupied by yeasts increased significantly after 24 h, followed by somewhat decrease after 72 h. In contrast, the areal porosity, inversely associated with the field of culture chamber decreased significantly after 24 h, followed by somewhat increase after 72 h. The length of edge line and the length of skeleton line increased significantly after 24 h, followed by somewhat decrease after 72 h.

Table 1. The parameters describing biofilm formation *in vitro* under static conditions on glass surface by *Candida albicans* oropharyngeal isolates

Parameter	The incubation time		
	h	*C. albicans*	*C. albicans*
		CA1	CA2
Areal porosity (%)	1	94.0±9.6	100±0.1
	24	61.0±5.7	44.0±12.9
	72	65.0±9.3	56.0±9.2
Length of edge line	1	49.60±74.73	0.40±0.27
[$mm(mm^2)^{-1}$]	24	182.77±53.71	231.67±77.72
	72	133.05±35.54	181.88±33.80
Length of skeleton line	1	33.07±61.45	0.16±0.12
[$mm(mm^2)^{-1}$]	24	105.78±36.60	133.71±35.57
	72	67.51±24.94	93.52±29.67
Thickness of biofilm (10^{-6} m)	72	13.3	66.42

The thickness of mature biofilms of *C. albicans* isolates, consisting of only blastospores, or blastospores and filamentous elements after 72 h incubation was 13.3 mm or 66.42 mm, respectively.

DISCUSSION

The formation of biofilm by *Candida* spp. has been demonstrated on a number of abiotic surfaces. The overall organization of *Candida* spp. biofilm is similar; mature biofilms consist of a dense network of yeasts and filamentous elements (hyphae or pseudohyphae). All species of *Candida* are able to form biofilm, but especially this ability possess yeasts belonging to species *Candida albicans* [1,7,8,10,11,13]. In this paper the non-invasive CSLM technique allowed to describe in details the structure of biofilm formed *in vitro* under static conditions on glass surface by oropharyngeal *C. albicans* isolates.

It is known that morphological transitions between yeast cells and hyphae or pseudohyphae is triggered after contact between fungal cells and surface. As found by other authors filamentous elements may have an important role in the structural integrity and the multilayered architecture of the mature biofilm, while dimorphism *per se* may not be an absolute prerequisite for the biofilm formation by *Candida* spp. [6,17]. As found here, *C. albicans* biofilm may consist of only blastospores or blastospores and filamentous elements. On the other hand, the thickness of *C. albicans* biofilm consisting of blastospores and filamentous elements is much higher than that consisting only of blastospores. According to the literature data the thickness of *C. albicans* biofilms ranged 25-550 µm, that of *C. tropicalis* biofilm – from 7 to 30 µm, while that of *C. parapsilosis* – from 75 to 125 µm [1,5,9,11,12,16,17,19].

The non-invasive CSLM technique enabled the quantitative analysis of *C. albicans* biofilm development on

a glass surface at various stages, *i.e.* during adhesion, the biofilm formation and its maturating. The calculated parameters describing the biofilm development, *i.e.* the areal porosity, the length of edge line, the length of skeleton line, indicate that the morphological reorganization within the biofilm occurred during its development. This may be due to some changes in the biofilm structure, *e.g.* coaggregation or disaggregation of microcolonies [3,4,6, 21]. According to our data, yeasts within the biofilm continued to proliferate during 72 h, but the parameters of biofilm appeared to be similar to those after 24 h. Similar observations were described by Andes *et al*. [1].

In conclusion, the presence different types of *C. albicans* biofilm architecture should be taken into account in the assessment of the effective methods used in yeast biofilm prevention and its eradication.

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