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Presence of Extracellular DNA in the *Candida albicans* Biofilm Matrix and its Contribution to Biofilms

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Abstract DNA has been described as a structural component of the extracellular matrix (ECM) in bacterial biofilms. In *Candida albicans*, there is a scarce knowledge concerning the contribution of extracellular DNA (eDNA) to biofilm matrix and overall structure. This work examined the presence and quantified the amount of eDNA in *C. albicans* biofilm ECM and the effect of DNase treatment and the addition of exogenous DNA on *C. albicans* biofilm development as indicators of a role for eDNA in biofilm development. We were able to detect the accumulation of eDNA in biofilm ECM extracted from *C. albicans* biofilms formed under conditions of flow, although the quantity of eDNA detected differed according to growth conditions, in particular with regards to the medium used to grow the biofilms. Experiments with *C. albicans* biofilms formed statically using a microtiter plate model indicated that the addition of exogenous DNA (>160 ng/ml) increases biofilm biomass and, conversely, DNase treatment (>0.03 mg/ml) decreases biofilm biomass at later time points of biofilm development. We present evidence for the role of eDNA in *C. albicans* biofilm structure and formation, consistent with eDNA being a key element of the ECM in mature *C. albicans* biofilms and playing a predominant role in biofilm structural integrity and maintenance.

Keywords *C. albicans* · Biofilm · Extracellular matrix · Extracellular DNA · DNase

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Introduction

Biofilms are structurally complex microconsortia of surface adhering cells embedded within an extracellular matrix (ECM) composed of substances produced and secreted by cells or derived from cell lysis [1]. The ECM contributes to the architectural preservation of biofilms by the maintenance of stable cell–cell and cell–surface interactions and acts as a protective barrier [2]. Although polysaccharides and proteins are the more extensively studied substances of biofilms ECM, other molecules such as lipids and nucleic acids play a crucial role on ECM functions [1]. In fact, extracellular DNA (eDNA) has been identified in the surrounding milieu as part of the ECM of biofilms formed by both Gram-negative

species such as *Pseudomonas aeruginosa* [3] and Gram-positive bacteria such as *Bacillus cereus* [4]. The proposed mechanisms implicated in eDNA release include: (1) cell lysis, (2) quorum sensing, and (3) excretion from DNA containing vesicles [3]. Bacterial biofilms eDNA characterization revealed that it comprises fragments of high molecular weight (around 30 Kb) [5] that differ from genomic DNA, as indicated by the different profiles exhibited upon restriction endonuclease treatments or randomly amplified polymorphic DNA analysis [6, 7]. Evidence for a role of eDNA as a structural component of bacterial biofilms ECM arose from studies showing that eDNA is required at the initial stages of biofilm formation [8, 9]. Additionally, independent studies evaluating exogenous DNA and DNase effect on biofilm cells antimicrobial susceptibility [5, 10] showed an association between eDNA and biofilms increased antibiotic resistance.

Despite the intensive research in the bacterial field, the investigation focusing on eDNA in fungal biofilms is scarce.

Candida albicans is a polymorphic fungus that causes opportunistic infections in humans and its ability to form biofilms is well characterized [11]. *C. albicans* ECM abundance was recently shown to be regulated positively by the glucoamylases Gca1 and Gca2 and the alcohol dehydrogenase Adh5 and negatively by the alcohol dehydrogenases Csh1 and Ifd6 [12]. *C. albicans* ECM consists predominantly of carbohydrates but also includes proteins, hexosamine, uronic acid, and phosphorous [13]. Consistent with the presence of phosphorous, circumstantial evidences point to the presence of eDNA in ECM. Specifically, mature biofilms treatment with 50 μg/ml of DNase resulted in 30% decrease of biofilm biomass, similarly to the observed for proteinase K treatments [13]. More recently, eDNA was extracted from *C. albicans* 72 h biofilm ECM within micrograms per gram of biofilm wet weight [14]. Additionally, it has been demonstrated that the presence of eDNA is a common feature of biofilms formed by other *Candida* species [14].

However, further studies are required to extend the knowledge on the contribution of eDNA to *C. albicans* biofilms structure and matrix composition. Here, we examined the presence of DNA in *C. albicans* biofilm ECM and the effect of DNase and the addition of exogenous *C. albicans* DNA on biofilm formation.

Materials and Methods

Strain and Culture Conditions

The *C. albicans* wild-type strain SC5314 was used in this study. Cells were stored at −70°C in 20% glycerol stocks and propagated by streaking a loop full of cells onto Sabouraud dextrose agar medium (BD, Franklin Lakes, NJ, USA) supplemented with 100 mg/l ampicillin (Fisher Bioreagents, Fair Lawn, NJ, USA) and incubating at 30°C for 24 h. These stocks were stored at 4°C for no longer than 2 weeks. For all experiments, batches (30 ml in 125 ml flasks) of yeast extract-peptone-dextrose (YPD) medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose] (US Biological, Swampscott, MA, USA) were inoculated with freshly grown yeast cells in an orbital shaker at 30°C for 24 h. Cells were harvested by centrifugation and washed in sterile saline. Cells were counted with a haemocytometer and dilutions were made to prepare standardized cell suspensions (1×10^6 cells/ml) in the appropriate prewarmed growth medium (see the next paragraph). Confirmation of the number and viability of cells was determined by plating serial dilutions on Sabouraud dextrose agar. Colony-forming units were counted after 24-h incubation at 30°C.

eDNA Estimation in *C. albicans* Biofilm ECM

Biofilm Formation Under Conditions of Flow

Three different influent media were used (1) RPMI-1640 supplemented with L-glutamine (Mediatech Inc., Herdon, VA, USA), sodium bicarbonate [0.2% (w/v)] and buffered with 0.165 M MOPS (Research Products International Corp., Mount Prospect, IL, USA), final pH 7, (2) YPD, and (3) yeast nitrogen base (YNB) (Fluka, St. Louis, MO, USA) supplemented with glucose [0.9% (w/v)] at final pH 7. The culture media used in the flow system were diluted in order to promote biofilm formation. Specifically, the chemically defined media RPMI and YNB were diluted 1:1, whereas the rich medium YPD was diluted 1:10. The media were supplemented with ampicillin 100 mg/l. Culturing conditions and apparatus were established as previously described [15]. Briefly, silicone elastomer (SE) strips (1 cm × 9 cm) (Cardiovascular Instrument Corp. Silicone sheets,

Wakefield, MA, USA) were washed twice with ultrapure water, sterilized by autoclaving and pre-treated overnight at 30°C with foetal calf serum (Sigma, St. Louis, MO, USA). *C. albicans* standardized cell suspensions (13 ml) were prepared using the same growth medium as the influent (1 × concentrated) and incubated with the SE at 37°C, 130 rpm for 90 min. After the adhesion period, the SE were transferred to the flow system and influent medium was set up at an initial flow rate of 0.75 ml/min [15]. Biofilms were formed at 37°C for 48 h. For subsequent analysis, two SE with biofilm were pooled together. Two independent experiments were performed for each growth medium condition.

Planktonic cultures (50 ml of standardized cell suspension per 125 ml flask; 1 × concentrated YPD, RPMI, or YNB) were grown at 37°C and 130 rpm for 48 h to validate the isolation of biofilm ECM (see the next paragraph).

Isolation of Biofilm ECM

ECM was isolated as previously described [16] with slight modifications. Briefly, after formation, the biofilms were transferred to polypropylene conical tubes (BD), resuspended in 10 ml of ultrapure sterile water and vortexed for 1 min. Next, biofilm cells were sonicated in an ultrasonic bath for 45 min, followed by a vortexing step of 2 min and centrifuged (2,000 rpm; 20 min). The supernatant fraction was recovered and filtered through 0.2-μm acrodisc syringe filters with Supor Membrane (Pall Life Sciences, Ann Arbor, MI, USA) that are low protein and nucleic acids binding membranes. Planktonic cultures were processed in parallel with biofilms during ECM extraction and aliquots were taken before and after the sonication step. The confirmation of planktonic cultures viability was performed by CFU counts in Sabouraud dextrose agar medium.

ECM Analyses

The DNA of the ECM fraction was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) (Invitrogen, Carlsbad, CA, USA) and then precipitated with isopropanol (1:1, v/v). After centrifugation, the pellet was rinsed with 70% ethanol and air dried. The pellet was dissolved in 20 μl of TE buffer [10 mM Tris–HCl (pH 7.5), 1 mM EDTA].

DNA was quantified using the Quant-iT^{TM} ds BR Assay kit (Molecular Probes, Invitrogen) according to the suppliers' instructions. This assay allows an accurate determination of DNA between 100 and 1,000 ng/ml. A λ DNA standard 45.7 μg/ml was prepared in TE buffer from a commercially available standard solution (Promega, Madison, WI, USA) and run in parallel with samples in each assay. Additionally, a blank (TE only) was also included in each assay. Samples were read in the Quibit^{TM} fluorometer (Molecular Probes, Invitrogen) and a new calibration was run for each assay. A minimum of one DNA measurement was performed for biofilm samples.

The protein content of the ECM fraction was estimated using the EZQ® Protein Quantification kit (Molecular Probes, Invitrogen). Standards for the assay were prepared in nanopure water (Biorad, Hercules, CA, USA) with ovalbumin supplied with the kit. Standard concentrations range was between 0 and 0.25 mg/ml in order to include sample concentrations. Fotodyne UV 300 transilluminator (Fisher) was used for UV measurements. Standard curves were generated for mean UV intensity vs. ovalbumin concentration. All measurements were made with three replicates.

Effect of DNase and Exogenous DNA Treatment on *C. albicans* Biofilm Formation

DNase I from bovine pancreas (Sigma) stock solutions were prepared before each experiment in 0.15 M NaCl supplemented with 5 mM of $MgCl_2$ (Merck, VWR, West Chester, PA, USA). The range of concentrations chosen was based on previously described titration data for bacterial species [17]. Working solutions of DNase in twofold increments ranging from 0.02 to 2 mg/ml were prepared in RPMI medium.

C. albicans genomic DNA, to be added as exogenous DNA for *C. albicans* cells treatment, was extracted with the MasterPure^{TM} Yeast DNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the suppliers' instructions. *C. albicans* DNA purity and concentration were estimated by measuring the A_{260} and A_{280} (Eppendorf Biophotometer, Eppendorf, USA). It has been previously described that in *C. albicans* stationary phase planktonic cultures eDNA levels are higher than 0.1 ng/ml [18]. In this sense, physiological and

supraphysiological concentrations ranging from 10 to 2,560 to ng/ml, with twofold increments, were chosen for this assay. Dilutions of the genomic DNA stock solution were prepared in RPMI medium.

For control experiments, DNase was heat inactivated at 95°C for 10 min and RNase (Sigma) stock solutions were prepared in TE buffer.

DNase Treatment and Exogenous DNA Addition at Different Stages of Biofilm Formation

Standardized cell suspensions (100 µl) were inoculated into polystyrene, flat-bottomed 96-well microtitre plates (Corning Inc., Corning, NY, USA) and incubated for 0, 1, 2, and 24 h at 37°C under static conditions. At 0-h (preincubation), DNase was added to the standardized suspension. At 1-, 2-, and 24-h of incubation the medium was removed and adhered cells washed two times with sterile PBS (10 mM phosphate buffer, 2.7 mM potassium chloride, 137 mM sodium chloride, pH 7.4) (Sigma). DNase working solutions were then added to *C. albicans* cells. The plates were incubated at 37°C for additional 24 h. The same procedure was performed for the addition of exogenous DNA to *C. albicans* cells. Positive controls for biofilm formation included RPMI only and RPMI plus 5 mM of $MgCl_2$ for DNase experiments. Negative background control for subsequent analysis consisted of RPMI medium only. Four independent assays with three to eight replicates for each condition were performed. The effect of DNase and exogenous DNA treatments was examined in terms of biofilm biomass changes using the crystal violet assay (see next paragraph).

As controls for DNase enzymatic activity, RNase, and heat inactivated DNase were added at 24 h as described previously. This experiment was performed once with eight replicates.

The effect of DNase and/or exogenous DNA on *C. albicans* viability and filamentation was examined in planktonic cultures and on preformed biofilms. For planktonic cultures, 5 ml of the standardized cell suspension plus (1) 5 mM of $MgCl_2$, (2) 2 mg/ml DNase, and (3) 2,560 ng/ml of exogenous DNA were incubated at 37°C, 130 rpm for 2 h. Viability was examined by CFU counts in Sabouraud dextrose agar medium. Filamentation was monitored by light microscopy using an inverted microscope (Westover Scientific, Mill Creek, WA, USA). These experiments were performed twice. The highest concentrations of DNase and exogenous DNA used in these experiments did not have an effect on *C. albicans* planktonic cells viability and filamentation.

For preformed biofilms, DNase ranging from 0.02 to 0.2 mg/ml was added to 24-h mature biofilms as described previously and incubated for an additional 24 h. Next, cells were removed with a micropipette tip, re-suspended in saline solution, and CFU analysis was carried out. Light microscopy examination of biofilms was performed in parallel. All the treatments were compared with the untreated (RPMI and RPMI plus 5 mM $MgCl_2$) controls.

Examination of the Effect of DNase Treatment and Exogenous DNA on Biofilm Formation

The effect of DNase and exogenous DNA on *C. albicans* biofilm biomass was estimated using the crystal violet assay. Briefly, the medium was removed and biofilms washed twice with PBS. Then, biofilms were stained with 50 µl of crystal violet 3 g/l [0.3% (w/v) crystal violet (Sigma), 5% (v/v) isopropanol, 5% (v/v) methanol, 90% (v/v) water] for 5 min. Afterwards, each well was washed twice with PBS, air dried, and destained with 100 µl of ethanol 100%. Next, 75 µl of the destaining solution was transferred to a new microtiter plate, and the A_{550} was measured (Benchmark Microplate Reader, Bio-Rad, Hercules, CA, USA). Samples exhibiting very intense colour, yielding "offscale" absorbance values, were diluted before performing a second absorbance reading.

Statistical Analyses

The association between the *C. albicans* 48-h biofilm ECM eDNA/protein content in the different experimental conditions was analysed using the chi-square test [19]. A statistical confidence interval of 95% was established. The comparison between (1) log CFU/ml before and after sonication and (2) each biofilm treatment and control condition was performed by two-tailed unpaired *t*-test (confidence interval 95%) using GraphPad Prism, version 5.00 software for Windows (GraphPad Software, San Diego, CA, USA). Differences were considered significant at $P < 0.05$.

Results

Presence of eDNA on *C. albicans* Biofilm ECM

Three different media were tested for their ability to promote the accumulation of eDNA in the ECM of 48-h *C. albicans* biofilms developed under flow conditions.

In the extraction of ECM, it was mandatory to ensure that there was no leak of cellular DNA during sample processing. To validate the ECM extraction process 48-h planktonic cultures were handled in parallel with biofilm samples. Before and after samples processing, aliquots were collected and CFU analysis was carried out. Difference between mean log CFU/ml before and after sonication was of: 0.00 ($P = 1.00$) for RPMI, −0.07 ($P = 0.39$) for YPD, and 0.00 ($P = 1.00$) for YNB.

As shown in Table 1, irrespective of which medium was used, eDNA was detected at measurable levels in ECM of *C. albicans* biofilms. Under the conditions used in this assay, the highest levels of eDNA in ECM were observed for biofilms formed using RPMI medium (3,045.4 ± 227.3 ng eDNA/mg of protein). These levels were 9-fold higher than those observed in the ECM of biofilms grown in YPD (Table 1; $P < 0.01$, Table 2). Both the absolute quantities of eDNA and the ratio of eDNA/protein were lowest in the ECM from YNB-grown biofilms when compared to biofilms grown in RPMI and YPD (Tables 1, 2).

This data shows that eDNA is a component of *C. albicans* biofilm matrix and suggests that its accumulation in the ECM is affected by culture conditions.

Effect of DNase Treatment on *C. albicans* Biofilm Formation

In order to examine the possible role of eDNA on *C. albicans* biofilm development, in the first set of experiments DNase was added to cells at different times (0, 1, 2, and 24 h) in the wells of microtiter plates and biofilms formation allowed to proceed for an additional 24 h (Fig. 1). The results of these experiments, assessed by a colorimetric assay for biofilm biomass showed that DNase did not exhibit major effects on *C. albicans* adherent cells at early time points during biofilm development (Fig. 1). DNase was also used to treat preformed biofilms (24 h) to determine whether different concentrations of this enzyme could affect the formed biofilms. As shown in Fig. 1, DNase showed a general inhibitory effect on *C. albicans* preformed biofilms. This was reflected by lower crystal violet readings at DNase concentrations higher than 0.03 mg/ml. For example, biofilms treated for 24 h with 0.13 mg/ml DNase showed a reduction of 40% in A_{550} readings when compared with the control ($P = 0.03$). Furthermore, preformed biofilms treatment with RNase and heat inactivated DNase at the same concentrations used for DNase did not display a decrease in biofilm biomass (data not shown), demonstrating that DNase activity is required for the observed effect.

Additionally, it should be noted that microscopic examination of biofilms, before washing, in terms of density and filamentation suggested that the DNase-treated biofilms (Fig. 2I-b) resembled the control biofilms (Fig. 2I-a). However, during the biofilm processing for the determination of biofilm biomass,

Table 2 Results of the statistical analysis of *C. albicans* 48-h biofilm ECM ng eDNA/mg of protein content (P values) obtained in the different experimental conditions

Media	P value
RPMI vs. YPD	< 0.01
YPD vs. YNB	< 0.01
YNB vs. RPMI	< 0.01

Table 1 *C. albicans* 48-h biofilm ECM eDNA and protein content

Influent medium	eDNA[a] (ng)	Protein[a] (mg)	eDNA/protein (ng/mg)
RPMI[b]	1,518 ± 822	0.48 ± 0.2	3,045.4 ± 227.3
YPD[c]	180 ± 160	0.42 ± 0.1	339.6 ± 265.6
YNB[b]	1.8 ± 1.6	0.10 ± 0.1	13.0 ± 8.8

DNA was extracted and further quantified. Protein content was directly assayed from the samples. Data presented represents mean ± mean deviation

[a] Obtained from biofilms developed under flow conditions, corresponding to 18 cm^2 of area; [b]used at 1:1 dilution; [c]used at 1:10 dilution

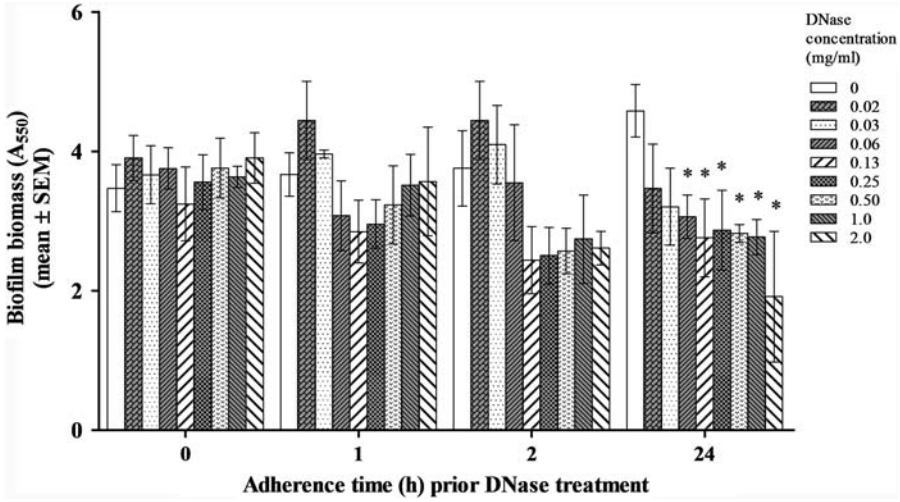

Fig. 1 Effect of DNase treatment on *C. albicans* biofilm formation. Different DNase concentrations (0, 0.02, 0.03, 0.06, 0.13, 0.25, 0.50, 1.00, and 2.00 mg/ml) were added to *C. albicans* cells at different times (0, 1, 2, and 24 h) of incubation in the wells of microtiter plates and incubated at 37°C under static conditions. The extent of biofilm formation was estimated by the crystal violet assay. Presented values are mean $A_{550} \pm$ standard error of mean of four independent experiments with three to eight replicates. Statistically significant differences (compared to biofilms formed in the absence of DNase) are indicated with an *asterisk*. (*$P < 0.05$)

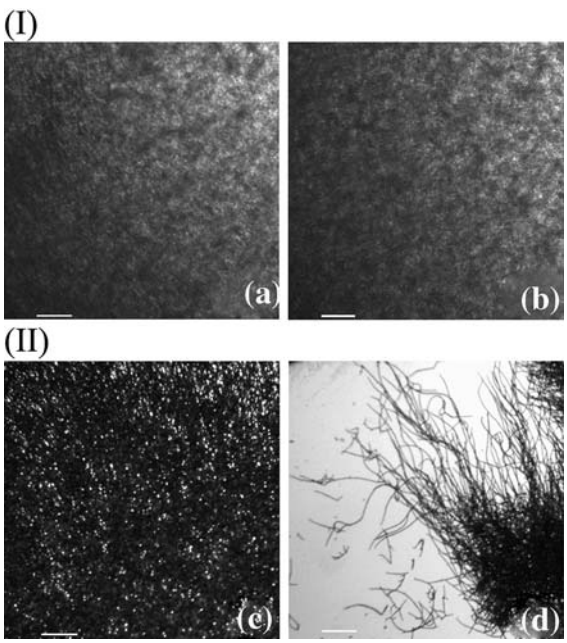

Fig. 2 Example of microscopy evaluation of the effect of DNase treatment on 24-h *C. albicans* biofilm formation. Microphotographs of cells in wells before (I) and after (II) aspiration of medium and subsequent washings with PBS for biofilms treated with 0 mg/ml (*a* and *c*) and 0.13 mg/ml of DNase (*b* and *d*). The *bar* in the picture represents 200 μm

the DNase-treated biofilms did detach from the microtiter plate surface (Fig. 2II-d), resulting in decreased A_{550}, in contrast to non-treated biofilms (Fig. 2II-c). Accordingly, the assessment of cell viability of prewashed biofilms did not reveal a detrimental effect of DNase on biofilm cells viability (compared with the control). For example, for 24-h treatment with 0.13 mg/ml DNase, the log CFU/well was 6.1 vs. 6.5 for control biofilms.

This suggests that the observed effect induced by DNase treatment is not due to changes in biofilm cells growth or filamentation, but rather due to a direct effect on the biofilm ECM which is important for biofilm integrity and maintenance.

Effect of Addition Exogenous DNA on *C. albicans* Biofilm Formation

To further substantiate the effect of eDNA as a structural component of biofilms, exogenous DNA was added to *C. albicans* cells. As shown in Fig. 3, the addition of exogenous DNA to *C. albicans* adhered cell populations did not affect further biofilm development. In contrast, addition of exogenous DNA, at concentrations higher than 160 ng/ml to

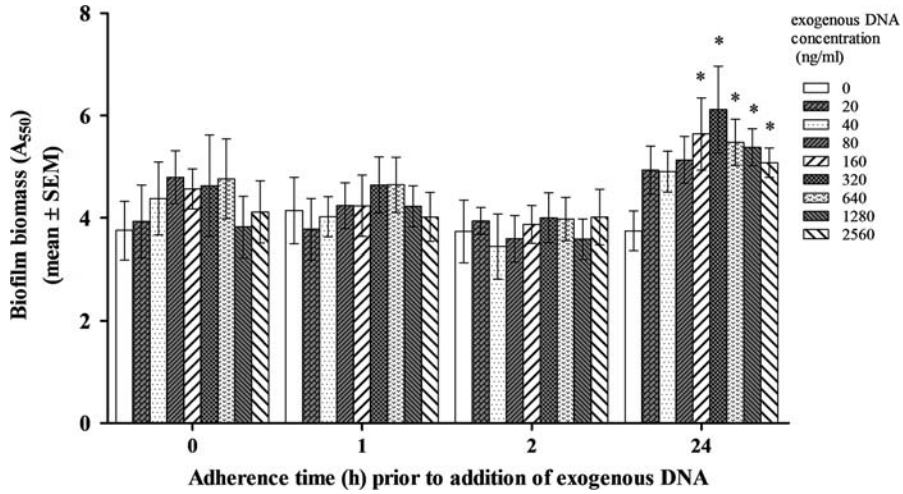

Fig. 3 Effect of addition of exogenous DNA on *C. albicans* biofilm formation. Different exogenous DNA concentrations (0, 20, 40, 80, 160, 320, 640, 1,280, and 2,560 ng/ml) were added to *C. albicans* cells at different times (0, 1, 2, and 24 h) of incubation in the wells of microtiter plates and incubated at 37°C under static conditions. The extent of biofilm formation was estimated by the crystal violet assay. Presented values are mean $A_{550} \pm$ standard error of mean of four independent experiments with three to eight replicates. Statistically significant differences (compared to biofilms formed in the absence of exogenous DNA) are indicated with an *asterisk*. (*$P < 0.05$)

mature biofilms led to increases in biofilm biomass, as indicated by the differences in A_{550} readings compared with control biofilms (grown in the absence of exogenous DNA) (Fig. 3). As an example, under the exposure to 320 ng/ml of exogenous DNA, biofilm biomass increased up to 63% in comparison with untreated biofilms ($P = 0.03$).

These experiments indicate that eDNA may contribute to the maintenance and stability of *C. albicans* mature biofilms, but is not required for the early stages of biofilms establishment.

Discussion

In recent years, there has been an increasing interest in the natural sources and target effects of the nucleic acids found outside the cells in several biological systems [20]. However, the study of eDNA in *C. albicans* biofilms has not been the subject of extensive research. Here, a series of experiments were designed to specifically address the contribution of eDNA to *C. albicans* biofilms ECM.

For this, we first determined the eDNA content from the ECM of 48-h biofilms using three different culture media, to ensure that eDNA is a component of ECM under different environmental growth conditions. Biofilms were developed in a flow model due to the predicted higher amounts of exopolymeric material that could be obtained when compared to biofilms developed under static conditions [15, 21]. Regardless of the growth medium used, eDNA was found to be a component of *C. albicans* ECM in all conditions used (Table 1). In this study, it was noticed that the amount of eDNA varied considerably with the growth medium used, with RPMI favouring the accumulation of eDNA in the ECM of *C. albicans* biofilms (Tables 1, 2). As the DNA content in the effluent medium was not determined, it cannot be discarded that biofilm cells grown in YPD released more eDNA compared with RPMI but the ECM amount/composition of RPMI biofilms promotes the accumulation of DNA. It should be noted that the association between ECM eDNA/protein content and growth medium was observed for biofilms formed in flow model.

The release of eDNA into the extracellular medium is not biofilm cells specific. It has been previously reported the release of DNA into the supernatant of *C. albicans* planktonic cells grown in RPMI and Hank's balanced salt solution [18]. The highest levels of eDNA attained in the stationary phase of growth have been suggested to result from cell death due to the nutrient depletion and toxic

metabolites accumulation [18]. We also determined the amount of free DNA in the supernatant of 48-h grown *C. albicans* planktonic cultures and observed the following hierarchy of eDNA accumulation relative to the amount of protein: YPD > RPMI > YNB (unpublished data).

In bacteria, it was proposed that quorum sensing is one of the mechanisms that contributes to increased release of eDNA into the environment [22]. Recently, the eDNA content of *C. albicans chk1/chk1* biofilm ECM was determined [14], which is a mutant strain known to be non-responsive to the *C. albicans* quorum sensing molecule farnesol [23]. The ECM of the mutant strain contained only slightly lower eDNA and protein levels/biofilm wet weight in comparison with the wild-type strain [14], suggesting that DNA release by *C. albicans* biofilm cells may not be a controlled step linked to cell–cell communication through farnesol.

Regardless the factors that promote eDNA accumulation, it was important to address the role of this ECM component in *C. albicans* biofilms. Circumstantial evidences have shown that DNA may play a structural role in *C. albicans* 48-h biofilms [13]. In this sense, we evaluated the impact of eDNA removal by DNase (Fig. 1), and addition of exogenous DNA (Fig. 3) at different stages of *C. albicans* biofilm formation. For the determination of the effect of these treatments on biofilms, a rapid and robust method, such as the 96-well plate model [24] that allows testing of multiple parameters in the same experiment was preferred. Furthermore, RPMI medium was the choice medium for the following experiments because it provides an optimal pH for DNase activity. Results from these series of experiments demonstrate that eDNA is an important component of *C. albicans* mature biofilms contributing to its maintenance and stability but is not required for the establishment of biofilms or during the very early phases of biofilm development (Figs. 1, 3).

Although it has not been determined how biofilms grown in 96-well plates compared to those developed under flow conditions the conjugation of the data obtained from the two models is consistent with eDNA being a key element of the ECM in mature *C. albicans* biofilms and playing a predominant role in biofilm structural integrity and maintenance. However, we note that eDNA role in *Candida* biofilms may be species/strain dependent since a previous study reported that *C. tropicalis* biofilm biomass is not affected by 50 μg/ml DNase treatment [13]. Data presented in this work also points to a differential role of eDNA among *C. albicans* and bacterial biofilms. In fact, for several bacterial species eDNA is required for the initial attachment as well as for subsequent early phases of biofilm formation, but only for some species DNA was found to be an important structural component of mature biofilms [8, 9].

In the last years, several lines of evidence suggested that in vivo eDNA has a functional activity in the regulation of host immune response. On one hand, it was demonstrated that the administration of *C. albicans* DNA to mice at specific time points prior to infection promoted mice survival [25, 26]. On the other hand, it was demonstrated that after *C. albicans* infection of non-neutropenic rabbits fungal eDNA was detected in the plasma within the first 24 h and increased over the course of disease [18]. This eDNA may be available for interaction with Toll-like receptor 9 (TLR9), an intracellular fungal DNA receptor, as evidenced by studies showing *C. albicans* eDNA activation of bone marrow-derived myeloid dendritic cells through the TLR9/Myeloid differentiation factor 88 signalling pathway [27], and the increased survival of TLR9 deficient mice upon challenge with *C. albicans* hyphae [28]. We can hypothesize that eDNA present on *C. albicans* biofilms may contribute to the modulation of host immune response.

Overall, this work reveals novel aspects of the role of eDNA on *C. albicans* biofilms, and properties of biofilm ECM that contribute to a better understanding of *C. albicans* biofilm lifestyle.

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References

1. Nobile CJ, Mitchell AP. Microbial biofilms: e pluribus unum. Curr Biol. 2007;17:R349–53. doi:10.1016/j.cub.2007.02.035.

2. Flemming HC, Neu TR, Wozniak DJ. The EPS matrix: the "house of biofilm cells". J Bacteriol. 2007;189:7945–7. doi:10.1128/JB.00858-07.
3. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, Kjelleberg S, et al. A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol. 2006;59:1114–28. doi:10.1111/j.1365-2958.2005.05008.x.
4. Vilain S, Pretorius JM, Theron J, Brozel VS. DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilm. Appl Environ Microbiol. 2009;75:2861–8. doi:10.1128/AEM.01317-08.
5. Tetz GV, Artemenko NK, Tetz VV. Effect of DNase and antibiotics on biofilm characteristics. Antimicrob Agents Chemother. 2009;53:1204–9. doi:10.1128/AAC.00471-08.
6. Steinberger RE, Holden PA. Extracellular DNA in single- and multiple-species unsaturated biofilms. Appl Environ Microbiol. 2005;71:5404–10. doi:10.1128/AEM.71.9.5404-5410.2005.
7. Bockelmann U, Janke A, Kuhn R, Neu TR, Wecke J, Lawrence JR, et al. Bacterial extracellular DNA forming a defined network-like structure. FEMS Microbiol Lett. 2006;262:31–8. doi:10.1111/j.1574-6968.2006.00361.x.
8. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS. Extracellular DNA required for bacterial biofilm formation. Science. 2002;295:1487. doi:10.1126/science.295.5559.1487.
9. Izano EA, Amarante MA, Kher WB, Kaplan JB. Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. Appl Environ Microbiol. 2008;74:470–6. doi:10.1128/AEM.02073-07.
10. Mulcahy H, Charron-Mazenod L, Lewenza S. Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog. 2008;4:e1000213. doi:10.1371/journal.ppat.1000213.
11. Ramage G, Saville SP, Thomas DP, Lopez-Ribot JL. *Candida* biofilms: an update. Eukaryot Cell. 2005;4:633–8. doi:10.1128/EC.4.4.633-638.2005.
12. Nobile CJ, Nett JE, Hernday AD, Homann OR, Deneault JS, Nantel A, et al. Biofilm matrix regulation by *Candida albicans* Zap1. PLoS Biol. 2009;7:e1000133. doi:10.1371/journal.pbio.1000133.
13. Al-Fattani MA, Douglas LJ. Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance. J Med Microbiol. 2006;55:999–1008. doi:10.1099/jmm.0.46569-0.
14. Paramonova E, Krom BP, van der Mei HC, Busscher HJ, Sharma PK. Hyphal content determines the compression strength of *Candida albicans* biofilms. Microbiology. 2009;155:1997–2003. doi:10.1099/mic.0.021568-0.
15. Uppuluri P, Chaturvedi AK, Lopez-Ribot JL. Design of a simple model of *Candida albicans* biofilms formed under conditions of flow: development, architecture, and drug resistance. Mycopathologia. 2009;168:101–9. doi:10.1007/s11046-009-9205-9.
16. Thomas DP, Bachmann SP, Lopez-Ribot JL. Proteomics for the analysis of the *Candida albicans* biofilm lifestyle. Proteomics. 2006;6:5795–804. doi:10.1002/pmic.200600332.
17. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, Molin S, et al. Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology. 2007;153:2083–92. doi:10.1099/mic.0.2007/006031-0.
18. Kasai M, Francesconi A, Petraitiene R, Petraitis V, Kelaher AM, Kim HS, et al. Use of quantitative real-time PCR to study the kinetics of extracellular DNA released from *Candida albicans*, with implications for diagnosis of invasive candidiasis. J Clin Microbiol. 2006;44:143–50. doi:10.1128/JCM.44.1.143-150.2006.
19. Fowler J, Cohen L. Statistics for ornithologists. 2nd ed. British Trust for Ornithology. 1995.
20. Vlassov VV, Laktionov PP, Rykova EY. Extracellular nucleic acids. Bioessays. 2007;29:654–67. doi:10.1002/bies.20604.
21. Hawser SP, Baillie GS, Douglas LJ. Production of extracellular matrix by *Candida albicans* biofilms. J Med Microbiol. 1998;47:253–6. doi:10.1099/00222615-47-3-253.
22. Spoering AL, Gilmore MS. Quorum sensing and DNA release in bacterial biofilms. Curr Opin Microbiol. 2006;9:133–7. doi:10.1016/j.mib.2006.02.004.
23. Kruppa M, Krom BP, Chauhan N, Bambach AV, Cihlar RL, Calderone RA. The two-component signal transduction protein Chk1p regulates quorum sensing in *Candida albicans*. Eukaryot Cell. 2004;3:1062–5. doi:10.1128/EC.3.4.1062-1065.2004.
24. Pierce CG, Uppuluri P, Tristan AR, Wormley FL Jr, Mowat E, Ramage G, et al. A simple and reproducible 96-well plate-based method for the formation of fungal biofilms and its application to antifungal susceptibility testing. Nat Protoc. 2008;3:1494–500. doi:10.1038/nport.2008.141.
25. Dimitrova P, Yordanov M, Danova S, Ivanovska N. Enhanced resistance against systemic *Candida albicans* infection in mice treated with *C. albicans* DNA. FEMS Immunol Med Microbiol. 2008;53:231–6. doi:10.1111/j.1574-695X.2008.00421.x.
26. Yordanov M, Dimitrova P, Danova S, Ivanovska N. *Candida albicans* double-stranded DNA can participate in the host defense against disseminated candidiasis. Microbes Infect. 2005;7:178–86. doi:10.1016/j.micinf.2004.10.011.
27. Miyazato A, Nakamura K, Yamamoto N, Mora-Montes HM, Tanaka M, Abe Y, et al. Toll-like receptor 9-dependent activation of myeloid dendritic cells by deoxynucleic acids from *Candida albicans*. Infect Immun. 2009;77:3056–64. doi:10.1128/IAI.00840-08.
28. Bellocchio S, Montagnoli C, Bozza S, Gaziano R, Rossi G, Mambula SS, et al. The contribution of the Toll-like/IL-1 receptor superfamily to innate and adaptive immunity to fungal pathogens in vivo. J Immunol. 2004;172:3059–69. doi:10.1128/IAI.69.4.2402-2406.2001.