Phenotypic evaluation of the effect of anaerobiosis on some virulence attributes of *Candida albicans*

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The current assumption that *Candida albicans* is a facultatively anaerobic organism has been widely accepted since its recovery from anoxic sites became common. However, the link between anaerobiosis and virulence remains uncertain. This study investigated the differential cell-surface hydrophobicity (CSH) using a hydrocarbon/water partition technique and analysed the differential secretion rates of secretory aspartyl proteases (Saps), esterase, chondroitinase and haemolysins of *C. albicans* strains recovered from periodontal pockets and non-periodontium-related intra-oral sites. For the enzymic tests, all strains from both sets were grown under aerobic and anaerobic conditions and the harvested cells were inoculated onto suitable normal or pre-reduced culture media in the presence or absence of molecular oxygen, respectively. The results showed that no variations were perceptible for CSH and chondroitinase ($P > 0.05$). The secretion rates of esterase and haemolysins strongly decreased in an anoxic environment ($P < 0.0001$). However, a consistent increment ($P < 0.0001$) in Sap secretion was detected when cultures were grown under anaerobic conditions. Based on these results, it is suggested that the oxygen concentration in the atmosphere surrounding cells exerts a variable influence on the virulence attributes of *C. albicans*.

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INTRODUCTION

The dimorphic fungus *Candida albicans* is the most commonly found eukaryotic micro-organism in the human oral cavity. The importance of this species in many oral mucosal diseases is well-known (McCullough *et al.*, 1996) and most of the niches colonized by this fungus exhibit an oxygen tension and redox potential (E_h) compatible with those detected in environmental conditions favourable for the growth of aerobic or, in some instances, facultative organisms. One of the pioneering efforts to evaluate the possibility of anaerobic growth of *C. albicans* is attributed to Szawatkowski & Hamilton-Miller (1978), who reported that aerobically cultivable strains could also grow in the complete absence of atmospheric oxygen. Nowadays, even the mating behaviour of *C. albicans* during anaerobiosis is known (Dumitru *et al.*, 2007).

C. albicans has the ability to regulate individual complexes and partition electron flux among the classical respiratory chain, the parallel respiratory chain and an alternative oxidase, which allows its cells to modify their energy and redox status in order to ensure survival (Ruy *et al.*, 2006). In the complete absence of oxygen, *C. albicans* cells may also use a fermentative pathway (Ogasawara *et al.*, 2006), which implies a reduction in cell division rates.

In recent years, interest in the lifestyle of *Candida* species in anoxic sites has increased. In the field of dentistry, many groups have shown that *C. albicans* is found in several oral niches that are not considered aerobic, such as periodontal pockets (Reynaud *et al.*, 2001), radicular dentinal walls (Siqueira *et al.*, 2002), infected root canals (Baumgartner *et al.*, 2000), sites of apical periodontitis (Waltimo *et al.*, 1997) and abscesses (McManners & Samaranayake, 1990). However, it remains uncertain whether these yeasts contribute to the development of pathological conditions.

In general, expression of the virulence factors of *C. albicans* has been evaluated almost exclusively under aerobic

Abbreviations: CSH, cell-surface hydrophobicity; Sap, secretory aspartyl protease.

conditions (i.e. environmental conditions with positive E_h values), with little investigation into the expression of fungal virulence attributes during anaerobiosis (Webster & Odds, 1987). Only fungal pleomorphism and resistance to antimycotics have been evaluated during anaerobiosis, with abundant phenotypic transition of yeast to hyphae being described when *C. albicans* is grown under strict anaerobiosis (Dahle & Olsen, 1991; Kaminishi *et al.*, 1994). Dumitru *et al.* (2004) found that *C. albicans* grown under anaerobiosis tended to become more resistant to azoles and polyenes than the same yeast strain grown under aerobic conditions.

Because *C. albicans* is found in intraoral tissues and niches under anaerobic conditions, it is plausible that this fungus may play a role in pathological conditions associated with this type of environment. Thereby, in order to predict the extent of this role, this study aimed to evaluate the effect of oxidoreductive environmental conditions on expression of virulence factors of oral *C. albicans*.

METHODS

***C. albicans* strains.** Strains 3A1, 13A3, 14A3, 15A2, 30A2, 30A4, 47A4, 55A3, 57A1 and 58A4 were recovered from periodontal pockets with a depth equal to or greater than 5 mm and were kindly provided by Professor José F. Höfling (Faculty of Dentistry of Piracicaba, Brazil). Strains B3, B8, C1, F1, F2, N1, N2, N3 and SC5314 were kindly provided by Dr Thein Zaw Moe (Oral Biosciences Unit, University of Hong Kong, Hong Kong) and were of diverse origins not related to periodontal disease. Strain ATCC 90028 was used as a reference strain.

Normal and reduced culture media. The culture broths used were YPD (2 % dextrose, 2 % peptone, 10 % yeast extract) and rYPD (YPD plus 0.1 % L-cysteine, pH 6). The semi-solid culture media used were BSAA (1 % yeast extract, 2 % glucose, 1.5 % agar, 0.2 % BSA fraction V, 0.25 % Protovit Plus, pH 4), rBSAA (BSAA plus 0.1 % L-cysteine), T80A (1 % peptone, 0.5 % NaCl, 0.1 % CaCl_2 , 1.5 % agar, 1 % Tween 80, pH 7.0), rT80A (T80A plus 0.1 % L-cysteine), CSA (1 % yeast extract, 2 % glucose, 1.5 % agar, 1 % chondroitin sulfate, 0.05 % BSA fraction V, pH 7.0), rCSA (CSA plus 0.1 % L-cysteine) and blood agar (3 % glucose, 1 % agar, 7 % sheep blood, pH 5.5).

Preparation of aerobic and anaerobic inocula. *C. albicans* cells were grown aerobically in 3 ml YPD at 37 °C. The culture was monitored until it reached an OD_{660} of 0.55 (Beckman DU 530 UV/VIS Spectrophotometer). The cells were then harvested, washed three times in 50 mM PBS (pH 6.5) and stored at 4 °C in 3 ml of the same buffer. This culture was designated the aerobic inoculum of *C. albicans*.

The following anaerobic cultures and manipulations were carried out in an anaerobic chamber (Coy Laboratory Products) with 85 % N_2 , 10 % CO_2 and 5 % H_2 . One hundred microlitres of aerobic culture was used to inoculate rYPD tubes. The cultures were incubated under anaerobic conditions and agitated at 150 r.p.m. at 37 °C. After growth, aliquots of 100 μl were taken and used to inoculate another set of rYPD tubes, which were incubated anaerobically at 37 °C with agitation at 150 r.p.m. until they achieved the optical density of a 7 MacFarland standard. These two-step inoculations were necessary to adapt the *C. albicans* strains to an anaerobic environment. The cells were washed twice with 0.1 % L-cysteine/0.9 % NaCl solution in sealed tubes and finally suspended in the washing solution at an OD_{660} of 0.55.

Semi-quantification of extracellular enzymes and haemolysins.

Plates with normal and reduced semi-solid culture media as described above were inoculated with four 5 μl spots of aerobic or anaerobic suspension onto the surface and left to dry on a laboratory bench or inside an anaerobic chamber, respectively. After incubation at 37 °C in a normal or anaerobic atmosphere for 10 days, the diameter of the hyaline zone around the colonies was measured using a digital caliper (UPM) with a resolution of 0.01 mm and an accuracy of ± 0.02 mm. Blood agar plates were evaluated at 48 h post-inoculation. Halo diameter measurements were carried out following the cardinal orientations N→S, E→W, NE→SW and NW→SE and mean values were used for statistical analysis. Each experiment was carried out in duplicate.

Cell-surface hydrophobicity (CSH). The CSH of *C. albicans* cells was measured using the method described by Sweet *et al.* (1987). Aerobic cells were grown on YPD for 24 h in a normal oxygen atmosphere at 37 °C. Anaerobic cells were grown on rYPD for 10 days in an anaerobic atmosphere at 37 °C. A loopful of each culture was centrifuged and washed twice with PBS (pH 7.0) and suspended in PBS at an OD_{520} of 1.0 ± 0.2 .

For each organism/atmospheric condition tested, 5 ml suspension was added to four glass test tubes, representing three tests and one control. Within a fume hood, 1 ml xylene (pro-analysis grade) was added to each test suspension. The tests and control tubes were placed in a water bath at 37 °C for 10 min to equilibrate. They were then vortexed for 30 s and returned to the water bath for a further 30 min to allow the immiscible xylene and aqueous phases to separate. The lower aqueous phases of the samples were removed carefully using a pipette and transferred to clean polystyrene tubes. Any traces of contaminating xylene carried over in the pipette or bound to the yeast were removed by attaching to the tube walls. The OD_{520} was measured as above.

Hydrophobicity values were expressed as the percentage reduction in optical density of the test suspension compared with the control. Thus the greater the change in optical density, the greater the shift in yeast cells from the bulk medium to the interface, i.e. the more hydrophobic the yeast isolate. Each assay was conducted in triplicate on two separate occasions.

Statistics. All experimental steps above were repeated in quadruplicate, on two different occasions, to give eight separate results. All data were evaluated in relation to their normal distribution by a Kolmogorov–Smirnov test and in relation to their homogeneities of variances by the Levene index. Differences were analysed using a Mann–Whitney U-test using the statistical package SPSS 13.0. A *P* value of 0.05 was assumed to be statistically significant.

RESULTS AND DISCUSSION

Enzymic and haemolytic activities (so-called Pz values) were determined by the ratio of the diameter of the colony to the total diameter of the colony plus the digestion/precipitation zone (Samaranayake *et al.*, 1984). Thus, a Pz value of 1 indicates no activity, whilst Pz < 1 indicates the degree of enzymic positivity. In order to facilitate the interpretation of our results, we used the value $1 - \text{Pz}$, so that the numeric results became greater as the enzymic activity increased.

All 20 *C. albicans* strains required 18–24 h to achieve an OD_{660} of 0.516–0.548 under aerobiosis. However, it was necessary to wait 72 h and a further 96 h to achieve the

same cell density after the first and second anaerobic inoculations, respectively.

Compiled data concerning the enzymic activities are presented in Fig. 1. All strains, independent of their origin or the atmospheric environment, secreted secretory aspartyl proteases (Saps). Non-parametric comparisons among the subgroups revealed that, under anaerobic conditions, candidal cells tended to secrete significantly higher amounts of Saps than under aerobic conditions ($P < 0.0001$). Saps are considered important molecules for the invasion and destabilization of tissues (Felk *et al.*, 2002; Hube *et al.*, 1997; Naglik *et al.*, 2003) and their secretion rates increase significantly when grown under anoxic conditions. Transcription of the genes *SAP4*, *SAP5* and *SAP6* is either up- or downregulated by the transcription factors Efg1 and Cph1 depending on environmental signals such as pH or temperature (Felk *et al.*, 2002). These two transcription factors are also directly involved in hyphal development (Biswas *et al.*, 2007). We postulate that they may also be implicated in the anaerobic overexpression of Saps, once enough data have been gathered supporting the growth of *C. albicans* as hyphae in this type of environment (Dahle & Olsen, 1991; Kaminishi *et al.*, 1994). Further studies involving *SAP4*–*SAP6* and other genes of the Sap family will elucidate this.

However, it cannot be generalized that anaerobiosis pushes the yeast to become more virulent, following a decrease in the secretion rates of other important factors such as

haemolysins and esterase ($P < 0.0001$ for both). In the particular case of haemolysins, Luo *et al.* (2001) showed that glucose added to aerobic blood agar can increase the detection of haemolytic activity in some strains. Shuster *et al.* (2004) described the phenomenon of ‘microbial alcohol-conferred haemolysis’ and showed that ethanol is readily oxidized to acetaldehyde with a strong haemolytic behaviour. Shuster *et al.* (2004) also found that this phenomenon is not observed when cells are grown under anaerobiosis. However, in a subsequent study, Shuster *et al.* (2007) presented a new premise based on forced overloading on the respiratory mode required to oxidize vast amounts of alcohol. This effect resulted in the production of high levels of hydrogen peroxide, a well-recognized haemolytic agent. Our results showed that all strains grown in the presence of oxygen secreted haemolytic products, whereas only a few (7/20) had this phenotype during anaerobiosis (Fig. 1). As we added glucose to the culture medium, it is reasonable that these few strains anaerobically fermented this substrate to ethanol, which, in turn, was readily oxidized to acetaldehyde (Shuster *et al.*, 2004), or had their alcohol dehydrogenase genes switched off, arresting the fermentative process at the aldehyde ending point. On plates incubated under normal levels of oxygen, pyruvate enters the tricarboxylic acid cycle and the electron-transport chain, driving all strains towards hydrogen peroxide production. We believe that enhanced haemolysis may not be attributed to anaerobiosis per se, as it was recently shown that blood cells can be stored better

Fig. 1. Differential activity of Saps, chondroitinase, esterase and haemolysins secreted by periodontium-related (Perio) and non-periodontium-related (Non-Perio) *C. albicans* strains grown under aerobiosis and anaerobiosis. Results are given as means $\pm$ SEM. Different letters denote P values less than 0.05. General data were obtained by the sum of results for periodontium-related and non-periodontium-related strains.

under such environmental conditions rather than under conditions of normal oxygen levels (Kriebardis *et al.*, 2007; Yoshida *et al.*, 2007).

Hube *et al.* (2000) studied the lipolytic behaviour of strain SC5314 in Tween 40 and found simultaneous expression of lipase and proteinase genes. Our results with cells growing in the absence of oxygen contrast with this, as, once the expression of Saps was increased, the esterase/lipase enzymes showed a diminished rate of secretion ($P < 0.0001$) or were completely inhibited (Fig. 1). It is likely that the anaerobic conditions alter the normal relationship between these two classes of enzyme. Hube *et al.* (2000) also showed that some lipase genes are always expressed during the yeast-to-hyphal transition under aerobic conditions. Again, our results revealed that the absence of oxygen generates a peculiar behaviour. Regardless of the fact that cells growing under anaerobiosis tend to grow as mycelia (Dahle & Olsen, 1991; Kaminishi *et al.*, 1994), the secretion of esterase exhibited a more divergent pattern than that expected for aerobiosis. Our belief is that the anaerobic conditions or the reductive environment guide lipolytic enzyme production towards alternative pathways.

Interesting, four periodontium-related strains showed no enzyme secretion when grown under anaerobiosis (data not shown). This finding corroborates the observations of Kurnatowska (1998), who showed that strains isolated from gingivitis and juvenile/adult periodontitis did not secrete, or only secreted discrete amounts of esterase, whilst strains isolated from mucous infection sites secreted a higher level of esterase. Therefore, it is possible that the periodontal pocket environment selects *C. albicans* strains whose esterase expression is inhibited in the absence of molecular oxygen.

The secretion of chondroitinase was not constant and only three strains secreted this enzyme. The atmospheric conditions did not exert much influence on the chondroitinase secretion phenotype once no differences were assessed for the summed data ($P = 0.2478$), although a discrete elevation in enzymic activity was observed when strains were grown in the absence of oxygen (Fig. 1). Few studies have shown the virulence potential of this enzyme in fungi (Coutinho & Paula, 2000; de Assis *et al.*, 2003; Shimizu *et al.*, 1995), and to our knowledge no data concerning gene expression, signal transduction or biochemical aspects of this enzyme in fungi are available.

Fig. 2 shows that CSH differences were not significant ($P = 0.9010$) except for the non-periodontium-related strains submitted to anoxia, whose mean value was less than that of the others ($P < 0.0001$). We suppose that, as such strains are adapted to grow in the presence of oxygen, when shifted to anoxic conditions their cell walls could not rearrange the hydrophobic proteins properly. However, this raises the question of the importance of this CSH reduction from 75.78–78.39 % to 68.62 %. Although statistically significant, this reduction did not appear to confer a less virulent phenotype on these strains.

Fig. 2. Differential CSH of periodontium-related and non-periodontium-related *C. albicans* strains grown under aerobiosis and anaerobiosis. Results are given as means $\pm$ SEM. Different letters denote P values less than 0.05. General data were obtained by the sum of results for periodontium-related and non-periodontium-related strains.

In conclusion, according to the results obtained in this study, it is clear that different atmospheric/redox conditions can exert a considerable influence over the virulence attributes of *C. albicans*.

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