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Molecular Characterization of Candida in the Oral Cavity and Factors Involved in Biofilm Formation and Virulence

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Summary, Conclusions, and Future Directions

The research described in this thesis addresses current issues related to oral *Candida* infections. Interactions of *Candida* with the oral microbiome were characterized and factors involved in biofilm formation and virulence were studied.

The oral cavity harbors a variety of mostly harmless, resident microorganisms, which are in a state of homeostasis with the host. This delicate balance may be disturbed by changes in the oral environment, e.g. induced by aging, illness, polypathologies (***e.g.*** diabetes and malignancies), polymedications (*e.g.* the use of antibiotics and/or corticosteroids), hospitalization, radiation, chemotherapy, immunosuppression therapies, malnutrition, hyposalivation, and the wear of a (partial) denture (Bodineau et al., 2009; Pfaller and Diekema, 2010). Imbalance of this homeostasis may result in an oral community that contains excessive pathogenic fungi, e.g. *Candida* spp. (De Resende et al., 2006; Kleinegger et al., 1996; Percival et al., 1991). Preventing fungal infections is a growing challenge in human medicine.

While various factors may influence the ecology of the mouth in older adults, we were able to generally characterize the oral microbial community wherein *Candida* species were represented (Chapter 2). We assessed the oral microbiome of 82 Dutch adults, between 58 and 80 years of age, using a next-generation sequencing approach, and quantified their *Candida* (ITS gene primers) and total bacterial (16S rRNA gene primers) load in saliva by qPCR. High *Candida* prevalence (98%) was found, with a median *Candida* load of 0.06% (ITS/16S). When specific *C. albicans* and *C. glabrata* primers were used, *C. albicans* was the most prevalent species (70%). Nearly half (46%) of the older adult population tested was positive for *C. glabrata*, and 40% harbored both *C. albicans* and *C. glabrata* (unpublished results). The prevalence of *C. glabrata* among the resident oral microflora in an older adult population may be of concern. Minor changes in the host coinciding with aging may create enhanced opportunities for the yeast to present its pathogenic properties. Moreover, the results from this study showed that microbiomes with a high *Candida* load were less diverse and had a distinct microbial composition predominated by acidogenic streptococci.

Subsequently, interactions between *Candida albicans*, *Candida glabrata* and *Streptococcus mutans* were studied using an *in vitro* biofilm model (Chapter 3). Mixed *Candida* or *Candida-S.mutans* biofilms were grown on different surfaces, which were either coated with saliva or not, in a medium containing glucose or sucrose. Biofilms were formed on hydroxyapatite (HA), polymethylmetacrylate (PMMA) and soft denture liner (SL) discs, the latter containing the antifungal agent undecylenic acid (1-5%). Confocal laser scanning microscopy was used to visualize biofilm structures and quantify hyphae formation. Viable *C. albicans* and *C. glabrata* biofilm counts were determined for each surface by colony forming unit (CFU) measurements, showing a decrease in the order HA>PMMA>SL. Although CFU counts on SL were lower than on PMMA, they were above background levels, indicating that *Candida* biofilms withstood the effect of the antifungal in SL. For almost all parameters tested in the study, higher CFU counts were observed for *C. glabrata* biofilms than for *C. albicans* biofilms or biofilms containing a mixture of the two species. This may be due to the smaller size and faster growth rate of *C. glabrata*. In some of the conditions tested, saliva coating resulted in a less dense biofilm structure harboring less *Candida* cells, possibly caused by antifungal proteins present in human saliva. The presence of *S. mutans* resulted in higher *Candida* CFU counts in some of the biofilms. In other biofilms, interactions between *C. albicans* and *C. glabrata* appeared to be synergistic. In the presence of glucose and *S. mutans*, *C. albicans* hyphae counts were lower than when the dual-species biofilm was grown in a medium containing sucrose. This is consistent with data from another group for single-species biofilms where proportions of yeast and hyphal cells have been shown to be dependent on the nutrient source (Maidan et al., 2005). Hyphae counts were also reduced in the presence of *S. mutans*, probably caused by competence-stimulating peptide, a quorum sensing molecule produced by *S. mutans* (Jarosz et al., 2009). Many more interactions between *Candida* and streptococci have been reported in the past (Holmes et al., 1996; Jenkinson et al., 1990; O'Sullivan et al., 2000). The various parameters investigated in our *in vitro* studies yielded results that underline how complex multi-species *Candida* biofilms are, and, therefore, *in vivo* interpretations should be taken with care.

Recently, *C. glabrata* has emerged as an important opportunistic pathogen due to the widespread use of immunosuppressive therapies, indwelling medical devices, and broad-spectrum antibiotics. *C. glabrata* has the ability to form

resilient biofilms and its high intrinsic resistance to commonly used azole antifungals poses a medical problem; it seriously complicates treatment of *C. glabrata* infections (Rodriques et al., 2014; Silva et al., 2012). Identifying specific (ecological) host niches for *C. glabrata* remains a challenge since this yeast seems to have the ability to rapidly adjust to environmental factors such as the host response, biofilm formation or antifungal drugs (Iraqui et al., 2005; Kaur et al., 2005; Rodriques et al., 2014; Silva et al., 2012). This potential pathogenic mechanism of rapid adaptation of *C. glabrata* has led to the intriguing question: what virulence factors are involved?

During adherence, biofilm formation, and infection of the human host by *Candida* the cell wall is involved. In *C. albicans*, morphological switching of a budding yeast form to an invasive hyphal form can occur. However, *C. glabrata* lacks this phenomenon. Morphologically and phylogenetically, *C. glabrata* is much more related to the non-pathogenic yeast *Saccharomyces cerevisiae* than to *C. albicans*. To get a better understanding of the cell wall structure of *C. glabrata* in relation to pathogenesis, biochemical experiments were performed (Chapter 4). Experiments revealed that the cell wall of *C. glabrata* contains the same basic components as *S. cerevisiae* and *C. albicans*. Mass spectrometric analysis identified 18 glycosylphosphatidylinositol (GPI)-modified cell wall proteins, carbohydrate-active enzyme Scw4, and four Pir proteins. Among the GPI cell wall proteins, four novel adhesin-like wall proteins (Awp), named Awp1, Awp2, Awp3, and Awp4 were discovered as well as Epa6, an adhesin that is involved in adherence to human epithelial cells and biofilm formation. A previous bioinformatics approach by Weig and colleagues, using an early release of the sequenced genome of *C. glabrata* strain ATCC2001, had identified 106 putative GPI proteins of which about 50% had features of adhesin-like cell wall proteins (Weig et al., 2004). In our study, using a more finalized version of the CBS138/ATCC2001 genome sequence, we performed a systematic screen to identify adhesin-like wall proteins (or protein fragments), defined as large modular GPI proteins with internal tandem repeats. Our screen showed that *C. glabrata* strain ATCC2001 contains at least 66 such proteins. Based on homology in the N-terminal effector domains, the putative adhesins could be divided into seven different groups, the largest group consisting of the well-studied epithelial adhesion protein (Epa) family. In strain ATCC2001, this family consists of 17 members whereas 23 members have been described for strain BG2, indicating a

large intraspecies genetic variability in the repertoire of (at least *EPA*) adhesin genes. In our studies, using exponential and stationary cell cultures of *C. glabrata* strains ATCC2001 and ATCC90876, Epa1 was not identified. Possibly, this is related to the observation by Kaur and colleagues who described that aspartic proteases are capable of removing Epa1 from the cell wall (Kaur et al., 2007). *In vitro* adhesion experiments in our study showed strain- and growth-phase-dependent cell surface hydrophobicity, which reflected the presence or absence of adhesin(-like wall protein)s. Because adherence is essential in biofilm formation, we anticipated increased incorporation of adhesins in cell walls of *C. glabrata* biofilms (Chapter 5). In *C. glabrata* strain BG2, expression of genes encoding Epa1 and Epa6 has been associated with biofilm formation (Cormack et al., 1999; Iraqui et al., 2005), whereas other *EPA* adhesin genes are reported to be repressed by chromatin-based silencing within subtelomeric regions (Castaño et al., 2005; De Las Peñas et al., 2003; Domergue et al., 2005; Iraqui et al., 2005). However, in biofilms, or other, niche-specific, conditions such as nicotinic acid limitation, inhibition of transcription of some adhesin genes by the silencing machinery may be alleviated (Domergue et al., 2005; Iraqui et al., 2005). To compare gene expression of known and newly identified adhesin genes between biofilm and planktonic conditions (cultured in two types of media to simulate a different environment), a quantitative real-time polymerase chain reaction (qPCR) was developed for these adhesin genes in the ATCC2001 strain. The results showed that most of the adhesin genes are significantly upregulated in biofilms in at least one of the culturing media. Interestingly, mass spectrometric analysis identified Epa3 and the novel adhesins Awp5 and Awp6, the latter only in cell walls isolated from cells in biofilms. Expression of adhesins and incorporation in the cell wall seems to be dependent on the genetic strain background and growth conditions. This supports the hypothesis that *C. glabrata* contains a large repertoire of adhesins, which governs a flexible, adaptive mechanism, and enables host tissue colonization under a variety of host niche-specific conditions (de Groot et al., 2013; Kraneveld et al., 2011).

In our final study, the phenotypic and genotypic diversity of different lineages of the reference strain CBS138/ATCC2001, obtained from different research laboratories, was analyzed (Chapter 6). Results from various cell wall-related phenotypic tests such as susceptibility towards the wall-lytic enzyme zymolase and other cell wall- or membrane-perturbing agents, and the cell wall

composition (relative content of glucan and chitin) correlated with specific karyotypic changes. Furthermore, changes in adherence capacity to plastic surfaces correlated with quantitative changes in adhesin gene expression rather than subtelomeric gene loss or differences in the number of tandem repeats within adhesin genes. These results emphasize the genomic plasticity of *C. glabrata* and imply that chromosomal abnormalities and functional adaptations may occur not only during infection and under antimicrobial therapy, but also under laboratory conditions without extreme selective pressures. This plasticity should be seriously considered when strains are shared between different laboratories or when phenotypic data are analyzed and compared.

All in all, the work described in this thesis contributes towards gaining a better understanding of oral *Candida* infections. Insights in *Candida* interactions with the oral microbiome were generated. High *Candida* load coincides with acidification of the environment, and this is probably the ecological factor that shifts the oral resident microbiome towards a microbiome with increased aciduric bacteria and, consequently, with a lower level of diversity. The question remains whether a high *Candida* load is a result of acidification of the oral environment by, for instance, high carbohydrate intake, or, originates from factors such as dry mouth, antibiotic- and or immunosuppression therapies in an aging- or immunocompromised population.

Future directions

The actual role of *Candida* as an opportunistic pathogen in (re)shaping the microbiome is complex and should be studied in future research focusing for instance on *Candida*-bacterial interactions in microcosm models *in vitro*. Although it will be difficult to extrapolate results from such models to the situation *in vivo*, this type of studies can be very useful in simulating and modulating oral interactions, studying biofilm structures, and testing or interfering with probiotics or antimicrobial compounds.

Tracking interactions in healthy and diseased populations, and in aging adults during longitudinal clinical trials, will give deeper understanding of natural interactions in real oral ‘microbiome life’ and will raise novel opportunities for intervention.

Furthermore, the thesis covers new progress made in the field of cell wall adhesins in the opportunistic pathogen *C. glabrata* and highlights the importance

of these proteins in establishment, progression and placticity of *Candida* biofilms and *Candida* infections. Future studies should be focused on functional characterization of novel putative adhesins. For instance, expressing full-length or specific domains of adhesin-like wall proteins on the cell surface of a non-adhering *S. cerevisiae* strain for gain-of–function studies (de Groot et al., 2013; Nobbs et al., 2010; Zupancic et al., 2008). Using this approach, adherence of *C. glabrata* to epithelial and endothelial cells was shown to be mediated, at least in part, by the proteins encoded by the *EPA* gene family (Cormack et al., 1999; Domergue et al., 2005; Frieman et al., 2002; Zupancic et al., 2008). Glycan microarray studies have been used to characterize carbohydrate-binding specificities of Epa1, Epa6 and Epa7 (Zupancic et al., 2008) and, more recently, high-resolution crystal structures have been used to study structure and ligand-binding specificities of adhesin Epa1 in detail (Ielasi et al., 2012; Maestre-Reyna et al., 2012). Elucidating structure, function, and specificities of other relevant adhesin-like wall proteins in *C. glabrata* will generate information about host substrata and/or host ecological niches where the fungus thrives, forms biofilms on, and their role in pathogenesis. This detailed information on adhesin-like wall proteins in *C. glabrata* may be of help in developing new anti-adhesive antifungal drugs to combat *C. glabrata* infection.

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