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Biofilm formation in invasive disease caused by fungus of the genus *Candida*

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ABSTRACT

Candida species are ranked as the fourth leading cause of nosocomial bloodstream infection. The association of the infection with several risk factors has been reported. The most predominant risk factor, however, is the presence of an intravenous catheter in patients, which provides an artificial surface for *Candida* spp. to form biofilms and subsequently initiate infection.

A total of 940 yeast isolates, mostly *Candida* spp., causing bloodstream infection in Sweden were collected between September 2005 – August 2006 and species identified through sequence polymorphisms in 40 nucleotides in the internal transcribed spacer region 2 (ITS2) of the ribosomal RNA gene obtained by pyrosequencing (paper I). Using this newly develop molecular approach, all isolates could be reliably identified down to the species level. In addition, intraspecies sequence variation in *Candida albicans* and *Candida glabrata* suggests subclassification of isolates. As *Candida* specification is associated with differences in susceptibility to antifungal drugs, accurate species identification aids treatment decisions. Pyrosequence analysis of the 40 nucleotides in the ITS2 region is fast and reliable, and can therefore contribute to the high quality management of patients with invasive fungal infections.

Biofilm formation of *Candida* spp. isolates was determined with a model mimicking the clinical conditions of the intravenous catheter surface through using a silicone elastomer, the host contribution through conditioning the surface with 10% human serum and the parenteral nutrition solution by a defined growth medium containing 10% glucose (paper II). Under these experimental conditions, biofilms of all *Candida* spp. with the exception of *Candida parapsilosis* were found to be composed of basal yeast cells. As a correlation between the amount of biofilm and the metabolic activity was observed, biofilm formation could be directly compared among *Candida* spp. isolates with isolates forming no, low and high biofilm. Biofilm formation was less prevalent in *C. albicans* isolates compared to non-*albicans Candida* spp. isolates. Thus, biofilm formation seems to be more significant for infections caused by non-*albicans Candida* species than by *C. albicans*. It is, therefore, possible that *C. albicans* uses mechanisms other than biofilm formation to cause bloodstream infection (paper II).

Among *Candida* spp., *C. parapsilosis* is the second/third most common cause of bloodstream infection. Biofilm formation of *C. parapsilosis* was highly variable among 33 epidemiologically

independent isolates (paper II and paper V). A nosocomial outbreak involving *C. parapsilosis* has been reported from a hospital in Southern Sweden (paper III). The clonal origin of the *C. parapsilosis* isolates was confirmed. All outbreak isolates robustly showed high level of biofilm formation with a compelx structure on three surface materials, while expression of major virulence factors was low (paper IV). This finding indicated the significance of biofilm formation of *C. parapsilosis* as a determinative factor to cause the outbreak infection.

Within 33 clinical isolates of *C. parapsilosis* causing bloodstream infection, two different biofilm architectures could be identified in the 15 isolates forming high level of biofilm (paper V). Of these 15 isolates, 11 showed a complex structure biofilm consisting of macro-colonies with a spider-like appearance composed of aggregated yeast cells and pseudohyphae, while four isolates formed monolayers of pseudohyphae. Surprisingly, biofilm formation of isolates with high biofilm formation including an outbreak isolate were independent of the transcription factor Bcr1, a major biofilm regulator, although *BCR1* deletion affected colony switching and cell morphology in those isolates. As novel phenotypes, *BCR1* regulated secretion of aspartyl proteinases and susceptibility to antimicrobial peptides irrespectively of the biofilm formation phenotype in all tested isolates of *C. parapsilosis* (paper V).

In conclusion, work in this thesis characterised biofilm formation in an extensive number of *Candida* spp. isolates causing bloodstream infection. Detailed analysis of biofilm development in *C. parapsilosis* revealed that high biofilm formation in *C. parapsilosis* is independent of the major biofilm regulator Bcr1. This finding will have an impact on the development of biofilm prevention strategies in *C. parapsilosis*.

LIST OF PUBLICATIONS

I. Pannanusorn S, Elings MA, Römling U, Fernandez V. **Pyrosequencing of a hypervariable region in the internal transcribed spacer 2 to identify clinical yeast isolates.** *Mycoses.* 2012 Mar; 55(2):172-80.

II. Pannanusorn S, Fernandez V, Römling U. **Prevalence of biofilm formation in clinical isolates of *Candida* species causing bloodstream infection.** *Mycoses.* 2013 May; 56(3):264-72.

III. Brillowska-Dabrowska A, Schön T**,** Pannanusorn S, Lönnbro N, Bernhoff L, Bonnedal J, Haggstrom J, Wistedt A, Fernandez V, Arendrup MC. **A nosocomial outbreak of *Candida parapsilosis* in southern Sweden verified by genotyping.** *Scand J Infect Dis.* 2009; 41(2):135-42.

IV. Pannanusorn S, Schön T, Morschhäuser J, Römling U. ***BCR1* independent biofilm formation of outbreak related *Candida parapsilosis* isolates from nosocomial bloodstream infections.** *Manuscript submitted.*

V. Pannanusorn S, Ramírez-Zavala B, Lünsdorf H, Agerberth B, Morschhäuser J, Römling U. **Characterization of biofilm formation and the role of *BCR1* in clinical isolates of *Candida parapsilosis*.** *Manuscript submitted.*

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LIST OF ABBREVIATIONS

ALS3	Agglutinin-like sequence 3
BCR1	Biofilm and cell wall regulator 1
BSI	Bloodstream infection
CDR2	*Candida* drug resistance
CFEM	Common in fungal extracellular membranes
C. albicans	*Candida albicans*
C. glabrata	*Candida glabrata*
C. krusei	*Candida krusei*
C. metapsilosis	*Candida metapsilosis*
C. orthopsilosis	*Candida orthopsilosis*
C. parapsilosis	*Candida parapsilosis*
C. tropicalis	*Candida tropicalis*
EAP1	Enhanced adherence to polystyrene 1
EFG1	Enhanced filamentous growth 1
HIV	Human immunodeficiency virus
HWP1	Hyphal wall protein 1
ITS1	Internal transcribed spacer 1
ITS2	Internal transcribed spacer 2
MDR1	Multidrug resistance
RBT1	Repressed By TUP1
ROS	Reactive oxygen species
rRNA	Ribosomal ribonucleic acid
SE	Silicone elastomer
TEC 1	Ty transcription activator 1
XTT	2, 3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt
ZAP1	*z*inc-responsive *a*ctivator *p*rotein 1

1 INTRODUCTION

1.1 *CANDIDA* SPP. AND CANDIDIASIS

The genus *Candida* consists of approximately 200 species and more than 17 species have been considered as medically significant (1). *Candida albicans* is the most frequently isolated and clinically most important species. Infections caused by species of *Candida* other than *C. albicans* such as *Candida tropicalis*, *Candida parapsilosis*, *Candida glabrata* and *Candida krusei*, however, are of increasing medical interest (2). *Candida* spp. are commonly found as normal flora in the gastrointestinal tract from mouth to rectum, as well as in the vagina, urethra and on the skin of humans (1). *Candida* spp. can also be isolated from animals and environmental sources. Being part of the normal flora, *Candida* spp. are therefore usually harmless for healthy individuals. However, an impaired host immune defence and an overgrowth of the yeast normal flora due to e.g. antimicrobial therapy in combination with an appropriate genomic background in the *Candida* spp. can lead to the development of serious opportunistic infections.

Candidiasis, infection caused by yeasts of the genus *Candida*, can occur all over the human body ranging from cutaneous infections of the skin and nails, to systemic infections of deep organs such as infections of the gastrointestinal tract, the respiratory tract, the urinary tract and the central nervous system (1). Most cases of candidiasis represent endogenous infections since these infections are caused by *Candida* spp. derived from the normal flora of the patients. Candidiasis also originates from exogenous sources by transmission of organisms from, for example, patient to patient and from healthcare workers to patient. The most typical example and well known form of candidiasis is "oral thrush" (oropharyngeal candidiasis), white patches or plaques on the tongue or other oral mucous membranes. The oral thrush is uncommon in healthy individuals, but it is the most predominant opportunistic infection in patients with acquired immunodeficiency syndrome (AIDS) (3, 4).

1.2 IDENTIFICATION OF CAUSATIVE AGENTS OF CANDIDIASIS

1.2.1 Biochemical identification

To determine yeast clinical isolates on the species level, there are several commercial identification kits available. For example, API 20C AUX (Biomerieux, France), Vitek ID (Biomerieux) and VITEK 2 (bioMe´rieux VITEK, Marcy l'Etoile, France). The identification is based on metabolic characteristics of yeasts to utilize/ hydrolyze a numbers of substrates employed in the kits such as the 19 carbon assimilation tests of API 20C AUX (5). This method is routinely used in many clinical microbiology laboratories worldwide, for rapid identification and ease of use (5-9). Drawbacks of the identification kits, however, are accuracy, the limitation of the database to identify newly emerging pathogens and a low discrimination potential to identify closely related species (10).

1.2.2 Molecular identification

The high rate of mortality and morbidity associated with invasive infections as well as the intrinsic differences in antifungal susceptibility on the *Candida* spp. level shows the urgent need to develop new methodologies to more rapidly and accurately identify the yeast pathogens causing disease down to the species level.

Several DNA based techniques have been developed in order to more rapidly achieve a higher accuracy in species identification, and to differentiate also closely related species (11-15). The molecular techniques also facilitate high-throughput identification and overcome the limitation of biochemical based methods to identify newly emerging pathogens. The most appropriate and widespread target for molecular identification of medically important yeasts are the ribosomal RNAs (rRNA). rRNA genes (18S rRNA, 5.8S rRNA and 28S rRNA) are highly conserved and separated by species-specific sequences of internal transcribed spacer regions (ITS1 and ITS2) (Figure 1). Therefore, several molecular identification methods have been developed based on detection of polymorphisms in this target region (12, 16). The general disadvantages of identification approaches based on molecular techniques are a high initial cost for equipment and expect expertise, which confines the techniques to the main reference microbiology laboratories.

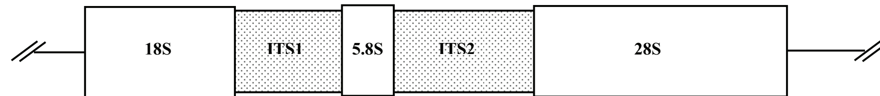

Figure 1. rRNA structure in fungi.

1.3 *CANDIDA* BLOODSTREAM INFECTION

1.3.1 Epidemiology

Candida spp. are responsible for most of the invasive fungal infections in humans ranking as the fourth leading pathogen causing bloodstream infection (BSI) in the United States (17) and one of the main causative agents of BSI worldwide (7, 9, 18-25). The incidence of candidemia as estimated from several epidemiological studies of nationwide settings is 0.17- 0.49 per 1,000 admissions (18, 26, 27). The rate of candidemia, however, is rising to 2.0-6.46 per 1,000 admissions in specific patient populations, such as intensive care unit patients (28) and premature births (18). Compared to other pathogens causing BSI, the highest mortality rate has been reported for *Candida* spp. which can be as high as 48% (29). *Candida* infections are associated with life-threatening underlying conditions of the patients, for example, surgical intervention, intensive care treatment, haematological malignancy, premature birth, cancer and HIV infection (18). Besides patient conditions, several physical risk factors associated with candidemia have been reported. Over 50 % of patients acquired candidemia were identified in patients with prior antibiotic therapy and central venous catheters (20, 30).

1.3.2 Species distribution

Various epidemiological studies performed in different geographic regions revealed the consistent result that *C. albicans* is the major leading causative agent of candidemia (Figure 2) (20, 23, 31-33). The frequency of infection of non-*albicans Candida* species, however, depends on the geographic region and the underlying conditions in the patient (18, 34). In general, the most common non-*albicans Candida* species, which cause infections are *C. glabrata, C. parapsilosis, C. tropicalis and C. krusei* (24, 28, 35). Bloodstream infections

caused by non-*albicans Candida* species are a rising concern as the occurrence of infection has increased over 50 % in the last two decades (36, 37). Most importantly, isolates of non-*albicans Candida* species are intrinsically resistant to fluconazole, a first line antifungal agent for treatment of invasive fungal infections (38, 39).

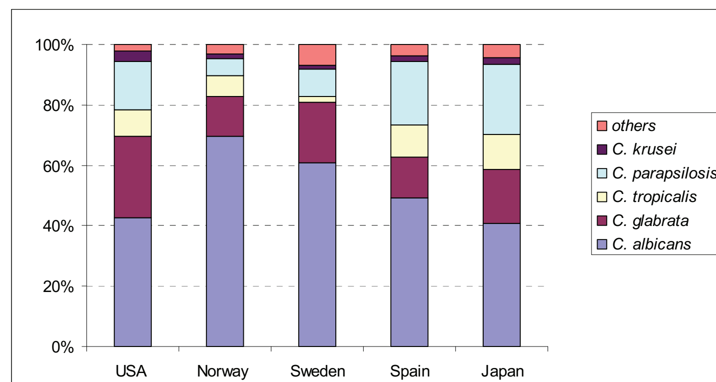

Figure 2. Distribution of *Candida* species causing candidemia by country (20, 23, 31-33).

1.4 *CANDIDA* BIOFILM

Biofilms are communities of cells developed on living tissues or on abiotic surfaces. Biofilm forming cell can also be attached to each other and are enclosed in a self-produced extracellular polymeric substance. This survival mode of growth allows cells to grow under hostile environmental conditions, for example, treatment with antibiotics and exposure to the immune response. Consequently, *Candida* biofilms have a significant impact on treatment in medical settings.

The presence of indwelling catheters or other medical devices in patients is a major risk factor of candidemia as the artificial device serves as a founding surface for the formation of a biofilm. About 90 % of candidemia cases resulted from a device-associated biofilm infection (40). The colonized device, moreover, serves as a reservoir from which the cells disperse to the bloodstream and consequently cause infection in distant organs.

1.4.1 *Candida* biofilm structure

The biofilm structure of *Candida* spp. varies from one species to the other. The biofilm of *C. albicans*, the main pathogenic *Candida* species, has been most extensively studied. A mature biofilm of *C. albicans* is mainly composed of cellular (yeast cells, hyphae and pseudohyphae) and noncellular elements (extracellular matrix) (41). As analysed from *in vitro* models and scanning electron micrographs (Figure 3a), yeast cells are the main component on the basement of *C. albicans* biofilms close to the surface. The middle layer of the biofilm is composed of hyphae. The top of the biofilm is mainly composed of budding yeasts and an extracellular matrix. In *in vivo* central venous catheter models (Figure 3b) (42), the biofilm architecture is similar to the one described *in vitro*. In addition, host cells can be embedded in the biofilm matrix (42).

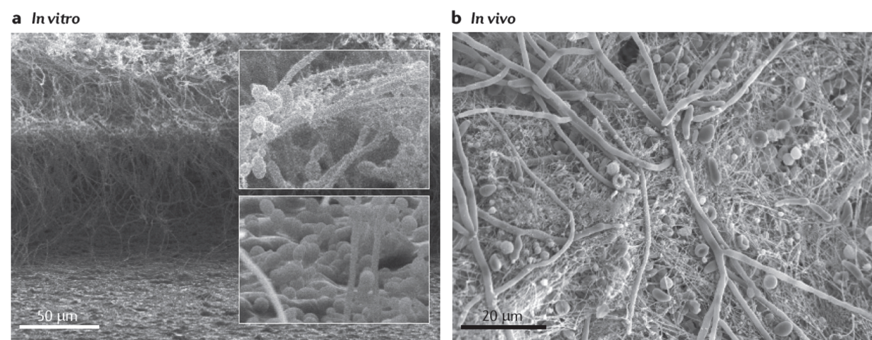

Figure 3. *C. albicans* biofilm structure *in vitro* and *in vivo* (42, 43).

1.4.2 *Candida* biofilm development

C. albicans is a main model for *in vitro* and *in vivo* biofilm developmental studies (42, 43). In general, *Candida* biofilm development can be divided into four sequential stages (Figure 4). Crucial factors of *C. albicans* required for the development of these steps have subsequently been identified.

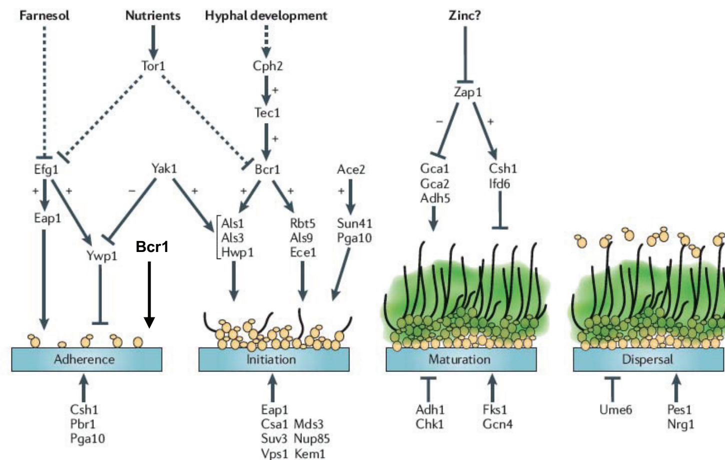

Figure 4. Biofilm development in *C. albicans* (43) with a slight modification at the adherence.

1.4.2.1 Adherence

The first step of biofilm formation is the attachment of yeast cells to a surface. *Candida* cells have cell wall proteins that mediate adherence to the surface. One important adhesin of *C. albicans* is Eap1 (enhanced adherence to polystyrene 1). A deletion mutant of *EAP1* displayed reduced adhesion to surfaces and was not able to form a robust biofilm. In addition, *EAP1* is upregulated in biofilms (44). Recently, the transcription factor Bcr1, a major biofilm regulator required for biofilm formation in *C. albicans* (45), has been shown to also be required for adherence of yeast cells to the surface as the first step to build up a biofilm (46).

1.4.2.2 Initiation

At the initiation stage, the adherent yeast cells proliferate and grow into hyphae or pseudohyphae which results in the formation of multiple layers of cells with different morphologies. At this phase, hyphal morphogenesis is required for the establishment of a robust biofilm. Several adhesion molecules are expressed predominantly by hyphae, for example, Als3 and Hwp1 proteins. These adhesins promote cell-cell or cell-substrate binding,

consequently mutants of these genes are defective in biofilm formation (47). The transcription factors Tec1 and Efg1 directly control hyphal development. *C. albicans* mutants defective in *TEC1* (45) or *EFG1* (48) are unable to form hyphae and consequently fail to form biofilm. Bcr1 is also involved in the initiation phase as expression of *BCR1* is upregulated in hyphae, however *BCR1* is not involved in hyphal morphogenesis. Instead, the Bcr1 transcription factor controls expression of many adhesins on hyphal cells, such as the Hwp1 and Als3 proteins. Therefore *BCR1* mutants of *C. albicans* retain the ability of hyphal formation, but fail to express adhesins on hyphal cells which causes a defect in biofilm formation.

1.4.2.3 Maturation

Extracellular polymeric substances are produced to enclose the mature biofilm, a complex network of cells including yeast cells, hyphae and pseudohyphae. The extracellular matrix of *Candida* biofilms is mainly composed of carbohydrates together with other constituents such as proteins, hexosamine, phosphorus, uronic acid and extracellular DNA (49, 50). The presence of the extracellular matrix strengthens the biofilm structure and protects biofilm cells from host immunity and antifungal treatment. The major carbohydrate component in the matrix is β- 1,3 glucan, which has an important role in antifungal resistance and host immune evasion (51, 52). The transcription factor Zap1 is a negative regulator of extracellular soluble β- 1,3 glucan. Deletion of Zap1 resulted in production of slime and a glistening biofilm appearance, but the biofilm biomass was not different compared to the wild type (53).

1.4.2.4 Dispersion

Dispersion is another key step of the biofilm development cycle (54). At this phase, cells are released from the biofilm out into the surrounding medium in yeast form. These cells can subsequently form a new biofilm. The dispersed yeast cells are responsible for candidemia and disseminate to distal organs. Dispersion is dependent on environmental factors, such as nutrition and pH. Comparison of dispersed cells from biofilms and age matched planktonic yeast cells showed that dispersed yeast cells had an enhanced ability to adhere to a surface, form a biofilm and cause endothelial cell damage, and had increased pathogenicity (54). These results indicate that dispersed yeast cells are associated with distinct phenotypes such as an increased virulence.

1.4.3 Environmental factors involved in biofilm formation

Candida cells possess a plethora of genetic mechanisms controlling development of a biofilm (55). Numerous studies have given a better understanding of the genetic control mechanisms of biofilm formation in *Candida* (45, 48, 55). On the other hand, many environmental factors can affect biofilm formation. Consequently, the impact of experimental parameters on *Candida* biofilm formation has been investigated (56-59). Some of the most notable parameters are described below.

1.4.3.1 Surface hydrophobicity and roughness

The type of the surface material has a pronounced effect on biofilm formation (56, 57). Physical properties of the surface material such as hydrophobicity and roughness are important for the adhesion of yeast cells. Several studies could correlate increased hydrophobicity and roughness to increased adhesion of cells to the surface (59, 60). The degree of hydrophobicity of the *Candida* cell surface also leads to a different adherence to an abiotic surface (60) and mouse tissue (61) suggesting that hydrophobic cells are more virulent than hydrophilic cells and important to initiate invasive disease (61). Hydrophobic interaction between cell and the material, therefore, increases adherence of yeast cells to a surface. An increased surface area as a consequent of increased roughness provides more area for biofilm formation. In addition, shear force is reduced on a rougher surface (62).

1.4.3.2 Medium and host conditioning

Commonly used media to assess *Candida* biofilm formation are, for example, rich medium – YPD (1% yeast extract, 2% peptone, 2% dextrose), synthetic define medium – YNB (yeast nitrogen base) and RPMI 1640 (Roswell Park Memorial Institute). Each medium contains different nutrients, supplements and pH which affect not only growth of cells, but also the architecture of biofilms and dispersion of cells from the biofilm (54, 63).

Pre-conditioning of the surface material with host body fluids such as serum, saliva and urine is commonly performed before biofilm formation is initiated. This procedure is performed in order to mimic infection conditions in the host (59, 64, 65) where a conditioning film is

formed on the medical device surface after contact with host body fluids. Pre-treatment of the surface leads to enhanced adhesion and biofilm formation (59).

1.4.3.3 Shear stress

In patients with a central venous catheter, biofilms frequently formed, and are exposed to shear stresses created by blood flow. These flow conditions can be induced experimentally by shaking or perfusion. The shear flow alters the behaviour of cells and consequently biofilm formation. It has been shown that production of the extracellular matrix is affected by flow conditions (58). Under shaking growth conditions, a higher amount of biofilm and the extracellular matrix were created compared to cultivation under static condition (58). In another study (66), comparison of biofilm formation showed a significantly lower amount of cells adhered to the surface and less metabolic activity under flow conditions compared to cultivation under static conditions after a short time interval of biofilm formation. However, after 24 h, the biofilm had a similar biomass and metabolic activity under both conditions. This finding suggested that cells withstanding the shear forces are selected and capable to form a mature biofilm (66).

1.4.4 Biofilm resistance against antifungal agents

Candida biofilm cells have been reported to have up to a more than 500-fold increased resistance against antifungal agents as compared to planktonic cells (67). It has been suggested that resistance against drugs is multifactorial involving several contributing factors (68). Factors which contribute to drug resistance in *Candida* biofilms are described below.

1.4.4.1 Physiological state of cells in biofilm

It has been suggested that differences in the physiological state of cells in biofilms compared to planktonic cells are associated with antifungal resistance. Physiological features of cells in biofilms such as the growth rate and an altered metabolism have been investigated for their role in resistance against antifungal drugs. A low growth rate of cells in the biofilm was proposed to be associates with resistance. However, Baillie and Douglas (69) showed that *C. albicans* biofilm are resistant to amphotericin B at different rates of growth. In bacteria, the presence of metabolically inactive (dormant) cells in biofilms contributes to tolerance to

antibiotics (70). Conversely, this metabolic heterogeneity of cells does not exist in *Candida* biofilms (68, 70-72) and also cells at basal layer exhibit a high metabolic activity (41). The results from these studies suggest a minor role of the physiological state of cells in biofilms in antibiotic resistance.

1.4.4.2 Restricted penetration of drug through the extracellular matrix

The presence of an extensive extracellular matrix is associated with a mature biofilm state. The extracellular matrix has been postulated to be a barrier prohibiting antifungals to access cells in biofilms. However, direct measurement of penetration of antifungal drugs demonstrated that antifungal drugs permeated through *Candida* biofilms as quickly as in 60 minutes (73). Other studies observed resistance against antifungals as early as 2 h after adhesion where an extensive extracellular matrix had not yet been produced (74, 75). Also, biofilms grown under static incubation with sparse matrix production were similarly resistant to antifungal drugs as biofilms grown with gentle shaking which leads to higher matrix production (76). These experimental evidences suggest that resistance against antifungal drugs is unlikely to be caused by the extensive production of an extracellular matrix (68).

1.4.4.3 Increased expression of drug efflux pumps

An increased expression of drug efflux pumps is associated with drug resistance in planktonic *C. albicans* cells (77, 78). Genes encoding drug efflux pumps in *C. albicans* include *CDR1*, *CDR2* (*Candida* drug resistance) and *MDR1* (multidrug resistance). Upregulation of efflux pump genes was observed in cells from biofilms compared to planktonic cells (75, 79). Deletion of these genes resulted in hypersusceptibility to antifungal drugs compared to parental strains (74, 79).

1.4.4.4 Alteration of membrane sterol composition

A significant decrease of the ergosterol level was observed in biofilm cells at intermediate and mature phase compared to cells in biofilms at an early phase (74). Ergosterol is a component of the fungal cell membrane and a target for azole and polygene drugs. Reducing the amount of ergosterol in cells in biofilms may be one of the mechanisms of biofilm-associated resistance.

1.4.5 Resistance to the host immune system

Candida biofilm forming cells are not only resistant to antifungal agents, but also to host immune mechanisms (51, 80). Within the protective extracellular matrix, mature *Candida* biofilms are more resistant to neutrophil killing compared to early biofilms. A study showed that production of Reactive-Oxygen-Species (ROS) by neutrophils which crucially contribute to killing of *Candida* cells is not activated by 24 h biofilms (51). The same study also showed that β-1,3 glucan, a main component of the extracellular matrix, is responsible for suppression of ROS production. This novel finding indicates that *Candida* biofilms evade killing by prevention to trigger a ROS response.

1.5 *CANDIDA PARAPSILOSIS*

C. parapsilosis was previously classified as a complex of *C. parapsilosis* consisting of 3 subgroups (group I, II and III). Several studies pointed out that the extent of the differences between these subgroups is significant to be sufficiently high to designate the subgroups into new species (81-85). *C. parapsilosis* group II and III isolates were therefore assigned as members of two new species, *Candida orthopsilosis* and *Candida metapsilosis*, respectively. The species *C. parapsilosis* is retained for group I isolates (86).

C. parapsilosis is a ubiquitous microorganism found in soil, water, air, and the marine and hospital environment (81, 87, 88). *C. parapsilosis* is also a human commensal and frequently isolated from skin and nails (89, 90). Recently, *C. parapsilosis* has been considered as an emerging pathogen due to the increasing number and spectrum of infections such as candidemia, endocarditis, meningitis and urinary tract infection (91). Unlike *C. albicans*, which is transmitted vertically as infections are caused by endogenous sources and colonization precedes infection, infection by *C. parapsilosis* is usually horizontally transmitted from exogenous sources such as medical devices, the air conditioner, and the hands of health care workers, which are contaminated by *C. parapsilosis*. Infections can occur without prior colonization (91-93).

C. parapsilosis is currently recognized as the second or third most common *Candida* species pathogen causing bloodstream infection (94). Of particular concern are infections in very low birth weight neonates and critically ill patients (95-97). It is notorious that bloodstream infections by *C. parapsilosis* are associated with the presence of a central venous catheter and the use of parenteral nutrition therapy with high a glucose concentration (98), which selects for *C. parapsilosis* to grow and form biofilms on catheters or other medical devices.

As *C. parapsilosis* is a commensal on the hands of health care workers, it is frequently responsible for nosocomial outbreaks (92, 99, 100). In paper III (99), we report the characterization of a nosocomial outbreak of *C. parapsilosis* infection in four patients admitted to a haematological ward. An internal investigation of the source of infection revealed the impairment in the routine procedures of maintenance of intravascular catheters as the most important factors causing this outbreak. Our subsequent study in paper IV shows a high level of biofilm formation in all isolates including consecutive isolates. Our results and the results of others (100) have strengthen the view that the capability to form a biofilm is a major virulence factor of *C. parapsilosis* associated with nosocomial outbreaks.

1.5.1 Biofilm architecture of *C. parapsilosis* biofilm

The biofilm architecture of *C. parapsilosis* is slightly different from *C. albican.* Only pseudohyphae instead of true hyphae are present in the biofilm, since *C. parapsilosis* cannot form true hyphae. However, the ability to form a biofilm among *C. parapsilosis* strains is highly variable (101), which is also reflected by different biofilm architectures presented in *C. parapsilosis*. Biofilm of *C. parapsilosis* is made up of multilayers of cells covered in an extracellular matrix. Biofilms consist either of aggregated yeast cells or yeast and pseudohyphal cells (67, 101). In paper II (102), we observed biofilm formation among *C. parapsilosis* isolates causing bloodstream infections to be highly variable with the architecture ranging from simple (aggregated yeast cells) to complex with a spider-like structure (aggregated yeast and pseudohyphal cells).

1.5.2 *Genetic control of C. parapsilosis biofilm*

Little is know about the genetic mechanisms controlling biofilm formation of *C. parapsilosis*. The recent development of genetic tools (103-105) and genome sequencing (106) facilitates genetic studies in order to explore *C. parapsilosis* biofilm development.

Like in *C. albicans*, pilot studies have shown that the transcription factor Bcr1 is required for biofilm formation in *C. parapsilosis* (104, 107). In *C. albicans*, Bcr1 is not involved in hyphae formation, but positively regulates several hyphal specific genes, the significant biofilm adhesins *HWP1* and *ALS3*. Therefore, *BCR1* mutant retains ability to form hyphae but the hyphae are functionally defective causing failure in biofilm formation (108). Similarly in *C. parapsilosis*, a *BCR1* mutant was shown to be defective in biofilm with pseudohyphae still present (104). However, *in C. parapsilosis*, the ortholog of *ALS3* is not regulated by Bcr1, while the ortholog of *HWP1*, a *RBT1*, is positively regulated by Bcr1(104).

Bcr1 regulates some of the genes of the CFEM family (common in fungal extracellular membranes) in *C. albicans* and *C. parapsilosis* (107). In *C. albicans*, the CFEM family consists of five genes, three (*RBT5*, *PGA10* and *CSA1*) are involved in a biofilm formation. The CFEM family of *C. parapsilosis* consists of seven genes, *CFEM1* – *CFEM7*. *BCR1* positively regulates expression of only *CFEM2*, *CFEM3* and *CFEM6.* However, *CFEM2*, *CFEM3* and *CFEM6* are not required for biofilm formation as mutants of each gene remain the ability to form a biofilm. Most likely other intact CFEM genes compensate.

1.5.3 *Biofilm formation and phenotypic switching*

Phenotypic switching in fungi is defined as the reversible alteration of colony morphology appearance which occurs at a higher frequency (10^{-2}-10^{-5}) than the somatic mutation rate (10^{-7}-10^{-8}) (109, 110). Three distinct switching systems, called the 3153A (111), the smooth-rough (112) and the white-opaque (113), were identified in *C. albicans*. It has been suggested that phenotypic switching in *C. albicans* may provide organism with the capacity to invade different host niches, to evade the host immune system and to change antibiotic resistance pattern (111). Phenotypic switching occurring at a high rate (10^{-1}-10^{-5}) has also been described for *C. parapsilosis* (114-117). In total, nine different colony morphotypes have been

reported from these studies. The cellular morphology of each colony morphology phenotype is also different and can vary from round or elongated yeast cells to pseudohyphae.

In *C. albicans*, cell surface adhesins are differently expressed between cells derived from white and opaque colonies (118). An altered ability to adhere to tissue cells and plastic has also been reported (119, 120). Different morphology phenotypes of *C. parapsilosis* show differences in cell morphology, agar invasion and biofilm formation (116). Concentric phenotype, a colony phenotype with pseudohyphae, generated the highest amount of biofilm and was most invasive on agar surface. It has been suggested that, like in *C. albicans*, *C. parapsilosis* undergoes phenotypic switching to adapt to different environmental niches.

As a part of results in paper IV, we report the colony morphology of 33 *C. parapsilosis* clinical isolates. A rough and rough/faint colony morphology is associated with the capability to form a biofilm.

2 SCOPE OF THE THESIS

In this thesis, the epidemiology of biofilm formation of yeast species causing bloodstream infection in Sweden during one year was analyzed. A subsequent emphasis was on the characterization of development and molecular analysis of biofilm formation in clinical isolates of *C. parapsilosis*.

2.1 Specific aims

Paper I:

The work in paper I aimed to identify species of yeast isolates causing bloodstream infection collected during one year from 2005-2006 in Sweden. To achieve this goal, a molecular identification method was developed based on pyrosequencing analysis of a variable 40 nucleotide sequence in the internal transcribed spacer 2 (ITS2) region of ribosomal RNA genes.

Paper II:

The work in paper II aimed to determine the prevalence of biofilm formation among *Candida* spp. causing bloodstream infection. Biofilm formation on silicone elastomer was quantified by estimation of the metabolic activity through XTT [2, 3-Bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt] reduction and simultaneous microscopy analysis.

Paper III:

In paper III, an outbreak infection of *C. parapsilosis* in a hospital in the south of Sweden was reported. The clonal origin of isolates was verified by multilocus sequencing typing (MLST), random amplified polymorphic DNA (RAPD) analysis and microsatellite typing.

Paper IV:

The work in paper IV aimed to determine and characterize the capability of biofilm formation of *C. parapsilosis* isolates from the outbreak reported in paper III. Biofilm formation was assessed on three different types of material, quantified by XTT reduction assay and qualitatively analysed by microscopy.

Paper V:

The aim of paper V was to in detail characterise biofilm development in clinical isolates of *C. parapsilosis*. An additional aim was to investigate the role of Bcr1, a major biofilm regulator, in the formation of distinct biofilm phenotypes of clinical isolates of *C. parapsilosis*. Additional physiological functions of Bcr1 were investigated.

3 EXPERIMENTAL PROCEDURES

The most important experimental procedures used in this thesis are briefly described here. All methodologies are described in the materials and methods section of each paper.

3.1 COLLECTION OF *CANDIDA* SPP. ISOLATES

A total of 940 yeast isolates causing bloodstream infection during September 2005 – August 2006 were sent to the Swedish Institute for Infectious Disease Control (SMI) from different medical laboratories in Sweden. The isolates were maintained in cryopreservative solution (Protect, Technical Service Consultants Ltd, Heywood, UK) at -150°C.

3.2 PYROSEQUENCING ANALYSIS

Pyrosequencing is a sequencing technology that can readily be adapted to high-throughput applications such as microbial identification and mutation detection. Biotin labeled PCR products of the 5.8S rRNA - ITS2- 28S rRNA region were prepared by PCR. Preparation of one strand of the PCR product labeled with biotin was performed according to the instruction of the manufacturer for vacuum prep tool (Biotage AB) and consequently used as template for pyrosequencing analysis. A local nucleotide database containing a 40 nucleotides long variable sequence of the ITS2 region of reference strains of medical important yeasts available in the public database was established. The 40 nucleotide sequences has been identified before by bioinformatic analysis to be sufficiently variable to provide discrimination on the species level. Results from pyrosequencing were BLAST searched against the local database in order to identify the species of yeast.

3.3 BIOFILM FORMATION

3.3.1 Surface material

In this thesis, biofilm formation on three different materials was studied. In paper II and V, silicone elastomer (SE) was used as a surface material as it represents the clinically most

relevant material. In paper IV and V, two additional materials, thermanox coverslip and polystyrene, were used to test the capability of *C. parapsilosis* isolates to form biofilms.

To condition the surface with host components, all surface materials were incubated with human serum diluted 10 fold with PBS at 37°C for 1 h and rinsed with PBS to remove residual serum.

3.3.2 Adhesion

An overnight culture of cells in Yeast Nitrogen Base medium (YNB) (6.7% Yeast nitrogen base (Difco), 1.5% L-asparagine) containing 10 % glucose was harvested by centrifugation, washed and the cell concentration was adjusted to 2×10^7 cells/ml in YNB medium containing 10% glucose. Cells were allowed to adhere to the conditioned substrate surface for 3 h at 37°C. Non adherent cells were removed from the surface by rinsing twice with 3.5 ml PBS. The amount of cells adhering to the surface was assessed by measurement of the metabolic activity. Adhesion of cells to the surface was simultaneously observed with microscopy.

3.3.3 Biofilm formation assay

Cells from an overnight culture adjusted to a final concentration of 10^7 cells/ml were inoculated into a well containing the material (Silicone elastomer, Thermanox coverslip) to assess biofilm formation or inoculated directly into the well of a 96-well plate to test biofilm formation on polystyrene. Cells were allowed to adhere to the surface for 3 h at 37°C and non adherent cells were removed by washing. The adhered cells on the surface were cultured in new medium to allow biofilm formation for 48 h at 37°C. The amount of biofilm was determined by metabolic activity measurement. Biofilm formation was simultaneously observed with microscopy.

3.3.4 Metabolic activity measurement

The amount of biofilm was determined by estimation of the metabolic activity of cells in biofilms. The metabolic activity was determined by quantification of the reduction of XTT.

XTT-menadione solution was added to PBS in the well of 24- well plate containing a washed surface with biofilm forming cells and the plate was incubated in the dark for 2 h at 37°C. Reduced XTT in supernatant was quantified spectrophotometrically at $OD_{490\ nm}$. The optical density of each sample was subtracted from the optical density of the blank control (PBS with XTT- menadione solution).

XTT is a tetrazolium salt which can be converted to a colored formazan product by mitochondrial succinoxidase and cytochrome P450 systems, as well as flavoprotein oxidases of viable yeast cells in biofilm. The colored formazan is a water soluble product and the amount of the formazan product can be spectrophotometrically measured in the supernatant (121).

3.4 OBSERVATION OF COLONY AND CELL MORPHOLOGY

To investigate colony morphology, cells were grown overnight in YPD medium at 30°C. After harvesting, cells were adjusted to a concentration of approximately 2×10^3 cells/ml, and 500 µl were plated on a YPD agar plate (14 cm diameter) and incubated for 5 days at 30°C. The colony morphology of a 5-day old colony was investigated with a stereo microscope (Leica MZ6). To investigate the cell morphology, cells of the 5 day-old colony were suspended in 10 µg/ml Calcofluor white and observed with a fluorescence microscope at 100× magnification. Calcofluor white is a fluorescent dye binding to β -1,4 polysaccharides such as chitin and cellulose.

3.5 SUSCEPTIBILITY

3.5.1 Antifungal susceptibility of *C. parapsilosis* biofilm

Three antifungal drugs with different mechanisms of action were used to determine the drug susceptibility of cells in a biofilm: fluconazole, amphotericin B and caspofungin. Fluconazole is a representative of the azole class of antifungal agents. Fluconazole inhibit 14α - demethylation of lanosterol in the ergosterol biosynthetic pathway. The polyene class of antifungal agents is represented by amphotericin B which binds to ergosterol, the major sterol of fungal cell membranes, and consequently cause leakage of cells. Caspofungin is a

representative of the echinocandins class of antifungal agents which targets proteins involved in cell wall β-1,3 glucan synthesis (122). Each drug solution was prepared according to the manufacturer's recommendation. Two-fold dilutions of drugs in YNB medium containing 10% glucose were prepared and a 48 h biofilm grown on SE was incubated with various drug dilutions for 48 h. The XTT assay was used to measure the viability of cells in the biofilm. MIC_{50} is defined as the minimum concentration of antifungal that reduced the metabolic activity of biofilm cells to 50% compared to the metabolic activity of the antifungal-free control.

3.5.2 Antimicrobial peptide susceptibility

Antimicrobial peptide susceptibility of *C. parapsilosis* isolates to peptide LL-37 and protamine sulphate was assessed using an inhibition zone assay. Six ml of 1% agarose solution in YPD medium containing *Candida* cell adjusted to $OD_{590\ nm} = 0.3$ were poured into a petri dish (9 cm diameter) and wells were punched into agarose. A total amount of the antimicrobial peptide of 10 µg was added into the well. The zone of inhibition was measured after the plate was incubated at 30°C for 24 h.

3.6 STRAIN CONSTRUCTION

The entire *BCR1* gene was deleted using the *SAT1* flipping strategy (123) (Figure 5). This strategy is based on the use of the dominant nourseothricin resistant marker cassette (*caSAT1*) for selection of integrative transformants. The cassette also contains a *Candida* adapted *caFLP* (flippase) gene encoding for the site specific recombinase which is required for excision of the cassette. To delete *BCR1* on both alleles, two rounds of transformation and cassette recycling were performed. The *BCR1* mutants and the complemented strain were verified by Southern blot hybridization.

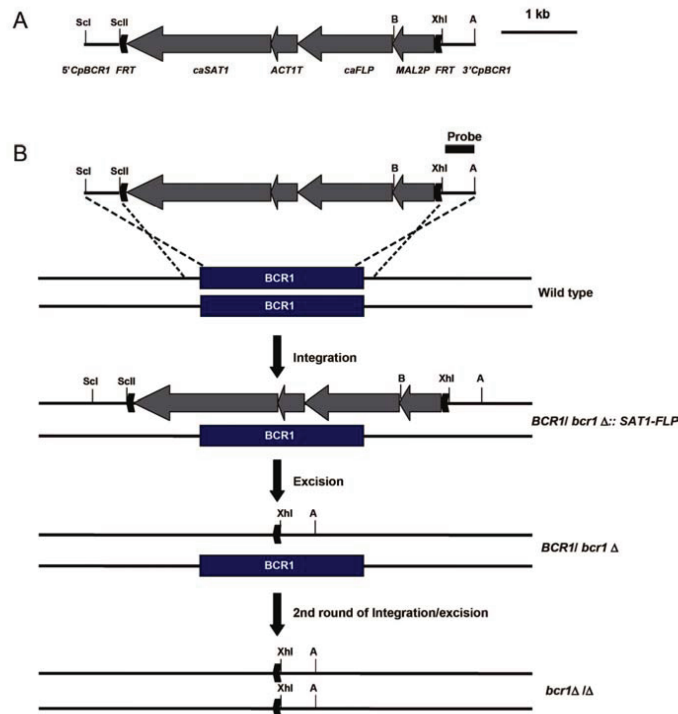

Figure 5. *BCR1* deletion strategy in *C. parapsilosis* clinical isolates. Structure of the disruption cassette based on *caSAT1* flipper (A). *FRT, FLP* recombination target sequence; *caSAT1*, nourseothricin resistance marker; *ACT1T*, *C. albicans ACT1* gene; *caFLP*, *C. albicans* adapted recombinase; *MAL2P*, the *MAL2* promoter (123). Diagram of construction of *bcr1Δ/Δ* strain (B). Restriction enzyme sites are located on the *caSAT cassette* as; ScI, SacI; ScII, SacII; B, BglII; Xh, XhoI; A, ApaI.

To reintroduce *BCR1* into the mutants, the entire *BCR1* gene including 450 bp of upstream sequence and 347 bp of downstream sequence was amplified and a complementation cassette was generated. The complementation cassette was integrated into one of the *bcr1*Δ alleles of the homozygous mutant, followed by recycling of the *SAT1* flipper cassette.

4 RESULTS AND DISCUSSION

4.1 IDENTIFICATION OF YEAST CAUSING BLOODSTREAM INFECTION

PAPER I

In this paper, we aimed to develop a molecular species identification approach for clinical yeast isolates superior to conventional identification methods. A total of 940 yeast isolates causing bloodstream infection during September 2005 – August 2006 were collected from 30 different clinical laboratories in Sweden at the Swedish Institute for Infectious Disease control (SMI). Identification of isolates was initially performed in the clinical laboratories by conventional identification methods available in each laboratory such as germ-tube test, assessment of colony morphology and colour on CHROMagar *Candida* medium (CHROMagar, Paris, France), morphology on cornmeal Tween 80 agar and different biochemical tests using the API 20C AUX System (bioMérieux, Marcy lÉtoile, France). Identification of isolates down to the species level was 89 % (835 of 940 isolates).

As a novel approach, we used pyrosequencing analysis of 40 nucleotides in the ITS2 region (5 bp of the 28S rRNA gene and 35 bp of the ITS2 region) to identify the clinical yeast isolates on the species level (Figure 6). Prior to experimentation, the 40 nucleotide region was bioinformatically identified to be sufficient to discriminate species of medically important yeasts. The primer for pyrosequencing was designed to obtain the first nucleotide of the 40 nucleotide region after the primer binding site which is located in a conserved region at the 5' end of the 28S rRNA gene.

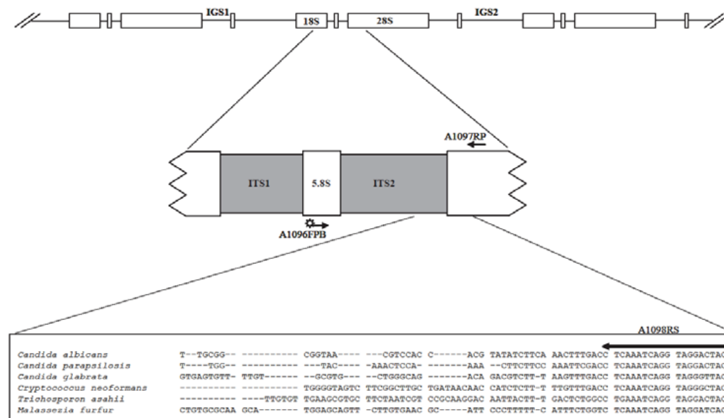

Figure 6. Diagram of the rRNA cluster of fungi and pyrosequencing approach (paper I). PCR product of the 5.8S rRNA -28S rRNA region is amplified by primers A1096FPB-A1097RP. ✡ indicates biotin label of the primer A1096FPB. Primer A1098RS is used for pyrosequencing analysis.

Pyrosequencing was successfully performed for all 940 clinical isolates. The comparison of the pyrosequencing results with our local reference nucleotide database identified all isolates on the species level. The isolates comprised 11 species of the genus *Candida* (931 isolates) and 3 species of other genus of yeasts (4 isolates of *Saccharomyces,* 3 isolates of *Malassezia* and 2 isolates of *Rhodotorula*). Of 940 isolates, 816 (87%) showed results identical to the conventional identification from the clinical laboratories. Discordant results for most of the remaining 124 isolates mainly resulted from the inability to identify the isolates down to the species level in the local clinical laboratories.

The sequence variability in the 40 nucleotide of ITS2 region was sufficient to reliably discriminate the closely related species of the *C. parapsilosis* complex (*C. parapsilosis, C. orthopsilosis and C. metapsilosis*) (Table 1). We also detected intraspecies sequence variation in the 617 isolates of *C. albicans* and the 164 isolates of *C. glabrata* which divided *C. albicans* and *C. glabrata* in three and two subgroups, respectively (Table 1). We could also detect these sequence polymorphisms in several sequences available in the GenBank

database. The physiological and biological importance of a potential subclassification of isolates needs to be investigated.

Table 1. Intraspecies sequence variation within 40 nucleotides of ITS2 region.

Yeast species (synonym)	**40 nucleotide ITS2 sequence**
Candida albicans	
C. albicans 1	GTCAAAGTTTGAAGATATACGTGGTGGACGTTACCGCCGC
C. albicans 2	.........................A..............
C. albicans 3	.........................A....C.........
Candida parapsilosis species complex	
C. parapsilosis	GTCGAATTTGGAAGAAGTTTTGGAGTTTGTACCAATGAGT
C. orthopsilosis	................~.......................
C. metapsilosis	................T.......................
Candida glabrata	
C. glabrata 1	GTCAAACTTAAAGACGTCTGTCTGCCCAGCACGCACAAAA
C. glabrata 2	.....................................G..

Although the sequencing readout from pyrosequencing is limited to 50-60 nucleotides, sufficient information can be generated for species identification, if appropriate target regions are selected (124-126). We showed here that 40 nucleotides in the ITS2 region are sufficient to provide information not only for species identification, but also subspecies discrimination. However, this region might not be appropriate for identification of complex species of *Cryptococcus* and *Trichosporon*.

Medical important yeasts show species-specific differences in susceptibility to antifungal agents (26, 127), therefore rapid and accurate identification of the causative agents of disease on the species level is needed. This is particularly important in patients with severe underlying life threatening conditions where appropriate antifungal treatment is especially urgent. Pyrosequencing is a fast and high throughput technology which, combined with appropriately chosen targets for molecular identification, could aid a significant improvement in treatment of patients with fungal infections.

4.2 PREVALENCE OF BIOFILM-FORMING *CANDIDA* SPP.

PAPER II

Biofilm formation is considered to be a virulence factor of *Candida* spp. Bloodstream infections caused by *Candida* spp. are often associated with contamination of medical devices with *Candida* isolates. However, the impact of biofilm formation as a virulence of *Candida* spp. infections is not entirely clear, in praticular as the number of isolates investigated in epidemiological studies was often limited. Therefore, we aimed to determine the prevalence of biofilm formation of *Candida* spp. from bloodstream infection. *Candida* isolates from 393 patients collected in the work for paper I were used.

To investigate biofilm formation *in vitro* related to a clinically relevant setting, biofilm was allowed to form on silicone elastomer pre-conditioned with 10% human serum for 1 h. Cells were allowed to adhere for 3 h to the silicone elastomer, non-attached cells were removed and incubation continued for 48 h at 37°C in YNB medium containing 10 % glucose. Under the condition tested, the isolates could be categorized as capable to form high, low and no biofilm based on the metabolic activity of isolates. Biofilm formation among these groups was statistically significant. Microscopic observation showed a qualitative correlation between the amount of biofilm formed and the metabolic activity.

It has been stated that the metabolic assay based on XTT reduction is not suitable for comparing biofilm formation between *Candida* species (67, 121). We addressed this issue by correlating the surface coverage by the biofilm with the metabolic activity using two representative isolates with a low and high biofilm forming capacity of different *Candida* species. The results showed that the metabolic activity could be correlated with the surface coverage between isolates of different species (Figure 7) demonstrating that the metabolic activity can be used to compare biofilm formation between different *Candida* spp.

Microscopic observation revealed that *Candida* spp. isolates which formed a high amount of biofilm displayed a biofilm structure mainly composed of dense multilayers of yeast cells, while the low biofilm consisted of cell communities of aggregated yeast cells with a less compact structure. Interestingly, *C. parapsilosis* isolates had more complex biofilm structure. Isolates with a high amount of biofilm showed the formation of macro-colonies with a

‘spider’ like appearance consisting of yeast cells and pseudohyphae. In contrast, in isolates forming a low amount of biofilm, the biofilm consisted of aggregated yeast cells attached to the silicone surface.

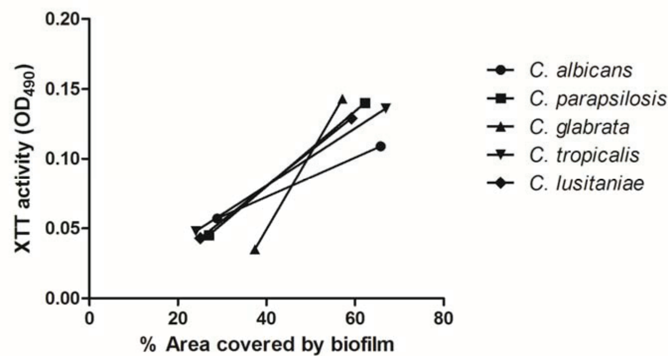

Figure 7. Correlation between the percentage of surface area covered by a biofilm and the metabolic activity assessed by XTT reduction for isolates of different *Candida* spp. (102).

Of all isolates of *Candida* spp., about 59 % were capable of forming a biofilm. Isolates of non-*albicans Candida* species (88.7%) were significantly more frequently positive for biofilm formation than *C. albicans* (40.3%). This result indicated that mechanisms other than biofilm formation are probably involved in bloodstream infections by *C. albicans*, a more pathogenic species, which more often causes bloodstream infections than non-*albicans Candida* species (6, 8).

4.3 CHARACTERIZATION OF OUTBREAK INFECTION ISOLATES OF *C. PARAPSILOSIS*

PAPER III AND IV

Cultures of blood samples of four patients (P1, P2, P3 and P4) in a haematological ward, Kalmar County Hospital were positive for *C. parapsilosis* within one month (July 2006). Since only one positive blood culture for *C. parapsilosis* had been recorded in the past five years, this was considered an outbreak of *C. parapsilosis* in this hospital as reported in paper

III. A common risk factor for infection in these four patients was the carriage of a permanent intravenous catheter. Subsequent episodes of reinfection could be detected in two patients (P2:2-4 and P4:2-3); in total nine isolates of *C. parapsilosis* were collected from this outbreak. Pyrosequencing analysis of 40 nucleotides in ITS2 region, a highly accurate methodology to determine the yeast species as reported in paper I, identified all isolates as *C. parapsilosis*. Sequencing of the entire ITS1-5.8S-ITS2 region did not show any sequence differences between the isolates. Analysis of polymorphic sites of *ACPL* (pro-acid protease) and *L1A1* (cytochrome P450 demethylase) (86) showed identical sequences among the outbreak isolates. The outbreak isolates showed a sequence difference at the *L1A1* polymorphic site compared to epidemiological unrelated control strains. RAPD analysis (115) and microsatellite typing of seven loci (loci A-G) (128) did not show difference between the isolates. These typing results indicated a clonal origin of all isolates besides isolate P4:3 which had been isolated six months after the first episode in patient P4. Microsatellite typing in this isolate differed in locus B, the locus with the highest degree of discriminatory power from the other isolates (128). Although the source of transmission could not be nailed down, internal evaluation revealed a lack of compliance with routine hygienic procedures by the clinical staff as a possible cause of the outbreak.

Biofilm formation is considered to be a virulence factor of *C. parapsilosis*. To investigate whether biofilm formation of *C. parapsilosis* is associated with this outbreak, we performed a biofilm formation assay of these outbreak isolates on three different materials, clinically relevant silicone elastomer, thermanox coverslip and hydrophobic polystyrene. All the outbreak isolates formed a high level biofilm with a complex spider-like macrocolony structure consisting of aggregated yeast cells and pseudohyphae on the three materials. We also tested the production of major virulence factors, secreted aspartyl proteinase and lipase, by these isolates. We found that the outbreak isolates secreted less proteinase and lipase compared to epidemiological unrelated isolates with a range of capacities from no to high to form a biofilm. Our results suggest that biofilm formation played relevant role in the outbreak. Moreover, biofilm formation of outbreak isolates was a stable characteristic during the outbreak infection.

In addition, a study on the role of *BCR1*, a major biofilm regulator of *C. parapsilosis* and *C. albicans* (45, 104), in biofilm formation of isolate P1 showed that biofilm formation of *C. parapsilosis* outbreak isolate is independent of the transcription regulator Bcr1.

4.4 BIOFILM FORMATION AND ROLE OF *BCR1* IN CLINICAL ISOLATES OF *C. PARAPSILOSIS*

PAPER V

Biofilm formation of 33 investigated clinical isolates of *C. parapsilosis* is highly variable as determined in paper II (Figure 8). Isolates were categorized according to their ability to form high, low and no biofilm. In paper V, we analysed the biofilm architectures of isolates more closely and identified two different biofilm structures within isolates forming high amount of biofilm. A biofilm with a complex structure composed of macro–colonies with spider-like appearance consisting of aggregated yeast cells and pseudohyphae (termed spider-like biofilm) was present in most of the isolates forming high amount of biofilm (11 of 15 isolates). A biofilm with a less complex structure composed of a monolayer of pseudohyphae (termed filament biofilm) was found in four isolates (Figure 8). Our observation indicated that clinical isolates of *C. parapsilosis* seemed to be locked at different stages of biofilm development. It would be interesting to investigate whether common regulators are non-functional in strains with a similar biofilm structure in future studies.

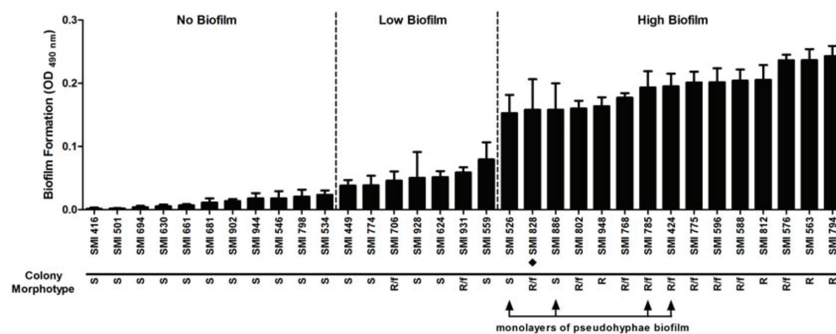

Figure 8. Biofilm formation on silicone elastomer by the 33 clinical isolates of *C. parapsilosis* after 48 h at 37°C. Colony morphology of the isolates is indicated as S (smooth), R (rough) and R/f (rough/faint). ♦ indicates the outbreak isolate (paper V).

It has been reported that biofilm formation of *C. parapsilosis* is associated with colony switching (116). A correlation between colony morphology of the isolates and the capability to form a biofilm was identified. Most of the isolates forming high biofilm exhibited a rough or rough/faint colony morphotype when grown on YPD medium at 30°C for five days. All isolates forming no biofilm exhibited the smooth phenotype. The rough/faint phenotype is a novel colony morphology type described for the first time in our study. This colony morphology was also observed in all isolates of *C. parapsilosis* causing outbreak infection as described in paper IV (Figure 9).

Figure 9. Smooth (left) and rough/faint (right) colony morphology observed in clinical isolates of *C. parapsilosis* (99).

The material surface can be a determinative factor for biofilm formation (59, 60). We found that the choice of material is critical for the capability of clinical isolates to form a biofilm in the case of the isolates with a high biofilm formation, but a less complex structure. All isolates with filament biofilm formed on silicone elastomer did not form biofilm on Thermanox and polystyrene (paper V). These findings highlight the importance of environmental factors on biofilm formation of *C. parapsilosis* and have implications for the epidemiology of biofilm forming strains of *C. parapsilosis*.

Biofilms of representative isolates with different biofilm formation capabilities were tested for susceptibility to amphotericin B, caspofungin and fluconazole. Although planktonic cells of

all isolates were sensitive to the drugs, isolates forming high and complex biofilm exhibited resistance to fluconazole ($MIC_{50} \geq 128$ µg/ml). The remaining isolates forming a high biofilm consisting of a monolayer of filaments and low biofilm formers were sensitive to the drug. Our results are in agreement with several studies which showed that resistance of *Candida* biofilm to fluconazole is common (129, 130). The biofilms of all isolates were considered to be sensitive to amphotericin and caspofungin. Therefore, amphotericin B and caspofungin are most likely good candidates to treat biofilm-related infection. However, the toxicity and side effects of amphotericin B to mammalian cells are a major health concern (122).

The transcription factor Bcr1 is reported to be a major biofilm regulator in *C. albicans* and *C. parapsilosis* (104, 108). Surprisingly, *BCR1* deletion showed a variable effect on 3 h surface adhesion and 48 h biofilm formation in representative clinical isolates with different capabilities to form a biofilm (Figure 10). The *bcr1*Δ/Δ mutants of strains forming low amount of biofilm and strains forming high biofilm consisting of a monolayer of filaments showed a significant decrease in biofilm formation after 48 h compared to wild type. In contrast, the *bcr1*Δ/Δ mutants of strains forming high biofilm with spider-like structure did not show an alteration of biofilm formation when compared to their respective wild types (Figure 10A). *BCR1* did also not affect early adhesion in those strains as the amount of biofilm of *BCR1* mutants and wild type were indistinguishable after 3 h adhesion (Figure 10B). These results demonstrated that biofilm formation of *C. parapsilosis* clinical isolates is both *BCR1*- dependent (Figure 10C) and *BCR1*- independent (Figure 10D). Loss of *BCR1*, however, led to a switch in colony morphology from rough/faint to crepe in isolates forming a high biofilm with a complex structure (with the exception of the outbreak isolate). Cells from the crepe colony appeared as elongated yeast cells and pseudohyphae compared to cells of the rough/faint morphotype which appeared mainly as round yeast cells. Investigation of additional phenotypes related to biofilm formation and virulence revealed that *BCR1* regulated asparatyl proteinase secretion and antimicrobial peptide susceptibility in clinical isolate of *C. parapsilosis* independent of biofilm formation. In conclusion, *BCR1* affects biofilm dependent and biofilm independent phenotype of clinical isolates of *C. parapsilosis*.

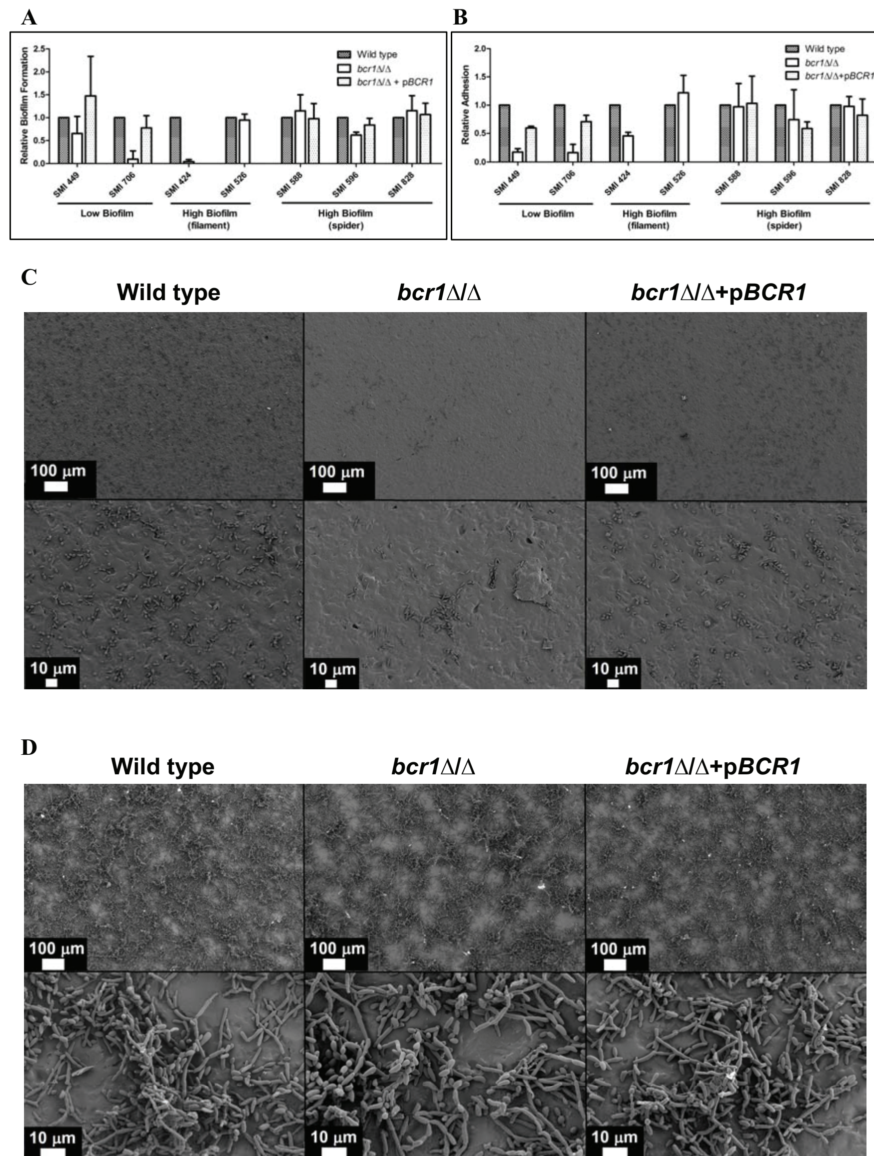

Figure 10. Effect of deletion of *BCR1* on biofilm formation of representative *C. parapsilosis* on SE square. Biofilm formation on SE square at 37°C after 48 h as determined by metabolic activity measurement (A). Initial biofilm formation of cells to the SE surface after 3 h (B). Scanning electron microscopy of SMI 706, its *bcr1* deletion mutant and complemented strain

indicated that SMI 706 formed a *BCR1* dependent biofilm (C) and of SMI 588, its *bcr1* deletion mutant and complemented strain indicated that SMI 588 formed a *BCR1* independent biofilm (D).

5 CONCLUSION AND FUTURE PERSPECTIVE

This work deals with species identification of *Candida* spp. isolates causing bloodstream infection and their capability to form a biofilm in a clinically relevant *in vitro* model. More than 900 isolates of *Candida* spp. had been collected from major clinical laboratories in Sweden during one year. A special emphasis was put on the phenotypic and molecular analysis of biofilm formation of *C. parapsilosis*.

Paper I: An accurate and rapid identification method for *Candida* species was developed based on the assessment of nucleotide polymorphism in 40 nucleotides of the ITS2 region obtained by pyrosequencing which were compared against a database of reference strains. Isolates could not only be identified down to the species level, but polymorphisms within the 40 nucleotides of the ITS2 region suggested subclassification of *C. albicans* and *C. glabrata* isolates.

The 40 nucleotides in the ITS2 region could not discriminate between all pathogenic yeast species. For example, in paper I, we have shown that complex species of *Cryptococcus* and *Trichosporon* could not be discriminated. For these species, the parallel analysis of other species discriminatory regions is suggested. In addition, the significance of the sequence polymorphisms within *C. albicans* and *C. glabrata* can be investigated in further studies. The database of the 40 nucleotide of medically important yeasts can be regularly updated in order to maintain the efficacy of this methodology to identify new emerging species.

Paper II: A significant difference in the prevalence of biofilm formation among *Candida* spp. causing bloodstream infection was observed. Under the experimental conditions in this study, isolates of *C. albicans* showed the least prevalence to form biofilms compared to isolates of non-*albicans Candida* species, suggesting mechanisms other than biofilm formation to be more significant for *C. albicans* to cause bloodstream infection.

This study is one of the most voluminous to investigate biofilm formation in *Candida* spp. Importantly, this study can be extended to include the clinical data of the patients. This would help to evaluate the association between biofilm formation and clinical factors such as the infection source, risk factors and outcomes. The categorization of isolates according to their

capability to form a biofilm can be associated with the pathogenicity of *Candida* spp. and with the capability of isolates to express additional virulence factors.

Paper III and IV: Robust high biofilm formation was a common factor of clonally related *C. parapsilosis* isolates derived from a two month outbreak occurring in a hospital in Southern Sweden. One common risk factor in the patients was the carriage of an intravenous catheter and transmission of *C. parapsilosis* between patients was traced back to impaired routine in hygienic procedures.

The biofilm of the isolates was composed of macro-colonies with aggregated yeast cells and pseudohyphae and independent of the major biofilm regulator Bcr1. As biofilm formation of clinical isolate of *C. parapsilosis* is variable, this finding suggests that biofilm formation played an important role in the outbreak infection.

Although our results showed that all isolates of *C. parapsilosis* causing the outbreak infection secreted less of the important virulence factors aspartyl proteinases and lipases compared to epidemiologically unrelated isolates, we cannot exclude that production of these virulence factors is not associated with the pathogenicity of bloodstream infection. Consequently, these *C. parapsilosis* isolates might take advantage of alternative virulence factors causing bloodstream infections. Whole genome sequencing of outbreak isolates and comparison to isolates from sporadic infection in combination with genetic and virulence studies will elucidate alternative virulence factors.

Paper V: We showed here and in paper II that biofilm formation was highly variable among 33 *C. parapsilosis* clinical isolates suggesting that the isolate are blocked at different stages of the development process of biofilm formation. Detailed analysis of mature biofilm revealed two different architectures among *C. parapsilosis* high level biofilm formers, an architecture composed of a monolayer consisting of pseudohyphae and a macro-colony type biofilm consisting of aggregated yeast cells and pseudohyphae. Surprisingly, our analysis showed that high complex biofilm formation of clinical isolates of *C. parapsilosis* was *BCR1* independent, although *BCR1* dependent switching of colony morphology was observed. *BCR1* also regulated secretion of aspartyl proteases and susceptibility to antimicrobial peptides in all isolates.

Work of paper V revealed that biofilm formation is a highly complex process in *C. parapsilosis* clinical isolates. Importantly, the transcription factor *BCR1* does not have a conserved function in biofilm formation among *C. parapsilosis* isolates. To elucidate central biofilm activators of high biofilm formers of *C. parapsilosis*, whole genome transcription analysis by deep sequencing in combination with a systematic deletion study of transcription activators elevated in biofilms would be the method of choice. This approach would probably not only reveal a single activator of biofilm formation. A recent systemic detection study of transcription factors showed that a regulatory network of six transcription factors controls biofilm formation in *C. albicans* and expression of target genes involved in biofilm formation is controlled by more than one regulator. Moreover, regulators also control the expression of each other (55).

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