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Studies of *Debaryomyces hansenii* killer toxin and its effect on pathogenic bloodstream *Candida* isolates

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STUDIES OF *DEBARYOMYCES HANSENII* KILLER TOXIN AND ITS EFFECT ON PATHOGENIC BLOODSTREAM *CANDIDA* ISOLATES

by

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STUDIES OF *DEBARYOMYCES HANSENII* KILLER TOXIN AND ITS EFFECT ON PATHOGENIC BLOODSTREAM *CANDIDA* ISOLATES

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Candida species are normal commensal organisms found in the skin, mouth, and gastrointestinal and genitourinary tracts of humans. *Candida albicans* is a normal component of the gut flora, and the oral and genital cavities. However, this organism is an opportunistic pathogen and it is one of the most frequently isolated species from patients with nosocomial fungal infections. *Candida* infections have grown in recent years due to antimycotics resistance and the extensive use of antibiotics. This has pushed scientists to look for alternative therapeutic agents to treat fungal infections.

Yeasts can produce toxic proteins called “mycocins” or “killer toxins” that can kill sensitive yeast species. The production of killer toxins is a phenomenon widely spread among yeast species. *Debaryomyces hansenii*, a yeast commonly found in cheeses, produces killer toxins that are able to kill the strains of *Candida albicans* SC5314 and *Candida tropicalis* NRRL-10985 at optimum conditions of 20°C and pH 4.5. The use of *Debaryomyces hansenii* as a biotechnological agent has been suggested, but the amount of toxins produced, and the genetic basis and mechanism of action of these toxins, remains a mystery.

To study the extent of pathogenic *Candida* species affected by this toxin, nineteen strains of *Debaryomyces hansenii* were tested for killer activity against *Candida* species, previously isolated from patients with bloodstream infections, by streak-plate agar diffusion bioassay. The killer activity of *Debaryomyces hansenii* killer strains was also tested against *Debaryomyces hansenii* non-killer strains. Additionally, to understand the nature of these toxins, 2x250 paired end Illumina sequencing was performed in 6 killer and 3 non-killer strains of *Debaryomyces hansenii* previously isolated from different types of cheeses. Numerous differences among the strains were found. Primers and protocols were designed to knockout presumptive genes and confirm their relationship with toxicity.

"Our greatest weakness lies in giving up. The most certain way to succeed is always to try just one more time."

-Thomas A. Edison

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Chapter 1

Killer yeast phenomenon: *Debaryomyces hansenii* mycocin production and its applications.

1.1 Killer Yeasts

Makower and Bevan first discovered the killer character in *Saccharomyces cerevisiae* in 1963 (Woods and Bevan 1968). Killer yeasts produce toxic proteins or glycoproteins (also called mycocins), and this characteristic has been found in more than 90 yeast species across more than twenty genera (**Table 1.1**) (Polonelli et al. 1991; Walker et al. 1995; Young and Yagiu 1978). Killer yeasts are able to kill sensitive yeast species by several biological pathways and the genetic basis of the killer phenotype can be chromosomal or cytoplasmic, encoded on double stranded RNA virus-like particles or linear double stranded DNA plasmids (Magliani et al. 1997). Moreover, as a self defense mechanism they possess intrinsic immunity to the action of their own mycocins (Schmitt and Breinig 2002). However, there exist situations where mycocin concentration prevails and this immunity is overcome, e.g., so-called suicidal mutants (K+ R–) (Polonelli et al. 1991).

Young stated that while all killer systems vary, they have a proteinaceous compound crucial to the mycocin activity that is common among them, and yeasts from the same species can produce several toxins with different modes of action (Young and Yagiu 1978). The reason behind killer toxin production is not fully understood; however, it is possible that killer yeasts produce these components when found in a competitive ecological niche to ensure survival of the species or strain (Polonelli et al. 1991). Hodgson agrees with Polonelli and states that these toxins might differ biochemically in response to ecological interaction with sensitive strains; i.e. killer strains will lose the ability to generate killer toxins if these are not essential for survival (Hodgson et al. 1995).

1.2 Killer toxins modes of action

The first step to achieve lethality on a susceptible organism is commonly a cell wall receptor on the sensitive cell that interacts with the killer toxin (usually chitin, β-glucans or mannoproteins) and suggestions exist about the existence of a second receptor on the plasma membrane that facilitates absorption (Magliani et al. 1997; Schmitt and Breinig 2006). Subsequently, they can achieve a lethal effect by different biological pathways. They can induce changes in the permeability of the membrane by forming an ion channel, leading to uncontrolled leakage of protons, potassium ions, amino acids and ATP molecules causing cell death (Marquina et al. 2002). Alternatively, they can release signals that will result in inhibition of DNA synthesis causing a lethal effect (Liu et al. 2015). Several authors explain the relationship between attacking the tRNA and cell death in the killer activity that *Kluyveromyces lactis* exhibits towards *Saccharomyces cerevisiae* (Frohloff et al. 2001; Jablonowski and Schaffrath 2007; Jablonowski et al. 2006). Additionally, a pathway blocking calcium uptake results in inhibition of cell growth and division (Liu et al. 2015). **Figure 1.1** illustrates several mechanisms of action of these killer toxins.

Killer toxins are usually temperature and pH dependent (Banjara et al. 2016; Magliani et al. 1997). Studies show that the common pH for killer toxin stability is between 2.0 and 4.5, and there exist some organisms for which toxin production is irreversibly inactivated above pH 5.0 (Young and Yagiu 1978).

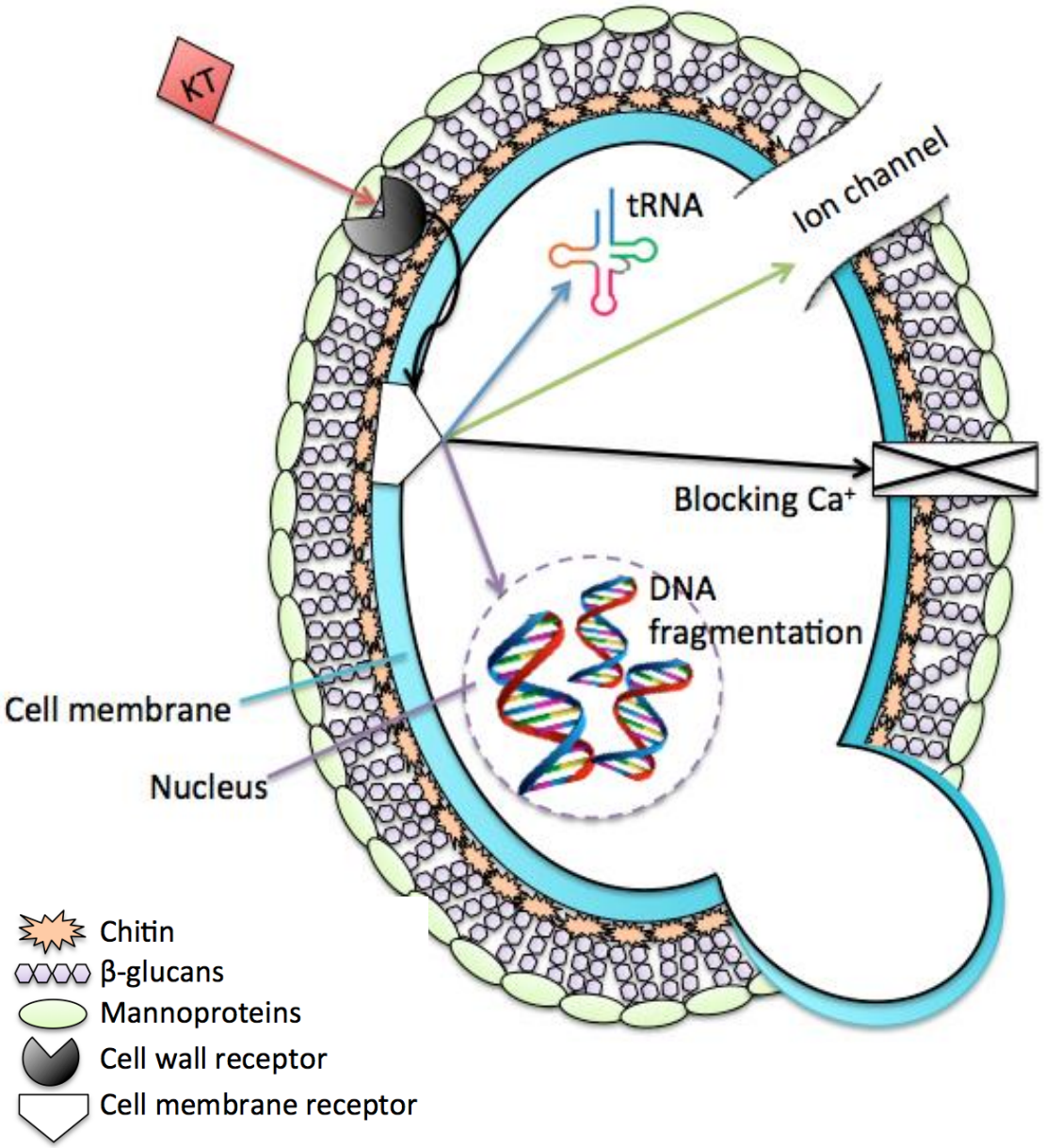

Figure 1.1 Schematic of different killer toxins modes of action (Liu et al. 2015).

1.2.1 *Saccharomyces cerevisiae* killer system

Saccharomyces cerevisiae, in which the killer phenomenon was first described, is the most thoroughly studied killer system. It produces several mycocins: three are cytoplasmically encoded on dsRNA virus-like molecules (K1, K2 and K28) and two are encoded on chromosomal DNA (KHS and KHR) (Magliani et al. 1997; Young and Yagiu 1978). Killer yeasts, as a self-protection mechanism, are normally immune to the action of their own mycocins (Bussey 1991; Schmitt and Breinig 2006). However, *S. cerevisiae* killer strains can present themselves as suicidal mutants (killer positive, resistance negative K+ R–) that produce killer toxins but exhibit a reduced immunity and when killer toxin concentration is higher than 100-fold it results in a lethal effect, as well as normal killer strains (K+ R+) and sensitive strains (K- R-) (Polonelli et al. 1991). Neutral mutants (K– R+) – resistant strains that do not produce killer toxins – also exist (Polonelli et al. 1991).

K1 toxin (19 kDa) is a disulfide-linked dimer (α and β) that acts on the susceptible species by a two-step mechanism. First, the toxin binds to the β-(1→6) glucans on the sensitive strain's cell wall; this step is dependent on a pH of 4.6. The second step consist of forming an ion channel that increases the permeability of the membrane, allowing leakage of protons and potassium ions followed by amino acids and ATP molecules, resulting in cell death (Bussey 1991; Magliani et al. 1997). K2 toxin presents the same mode of action as K1 although it is a different two-subunit protein with a molecular weight of 21.5 kDa. However, killer toxin K28, which forms a heterodimer and has a molecular weight of 21.5 kDa, possesses a different mode of action. The first step consists of binding to the α-1,3-mannoproteins (Schmitt and Breinig 2002). The

second step involves stopping DNA synthesis by blocking G1 phase, which provides a lethal effect (Marquina et al. 2002). The modes of action for toxins KHR and KHS are unclear, but Magliani indicates the possibility of KHS working by ion-channel formation similarly to K1 and K2 (Magliani et al. 1997).

1.2.2 *Kluyveromyces lactis* killer system

Plasmids in yeasts are either dsRNA, circular DNA or linear DNA (Fukuhara 1995). Gunge et al. discovered the *Kluyveromyces lactis* killer phenotype while searching for plasmids in yeasts (Gunge et al. 1981). *K. lactis* killer strains have been found to exhibit the killer phenotype against several genera and species including *Saccharomyces*, *Candida* and *K. lactis* non-killer strains (Gunge et al. 1981).

K. lactis possesses two plasmids, pGKL1 and pGKL2, with pGKL2 being necessary for pGKL1 maintenance and replication. The killer toxin produced by this organism, as well as the immunity region, are encoded on the linear double stranded DNA plasmid pGKL1 (Fukuhara 1995). The killer toxin consists of a 156 kDa trimer (**Figure 1.2**) with subunits α, β and γ (Liu et al. 2015). Four open reading frames (ORFs) have known functions in pGKL1 (**Table 1.4**), with ORF2 and ORF4 coding for the predecessor of the mycocin and ORF3 providing the immunity determinant (Magliani et al. 1997). The mode of action of this toxin is not well comprehended. However, it is known that the toxin binds to the chitin component of the cell wall in the susceptible strain and results in cell cycle arrest at the G1 phase (Magliani et al. 1997).

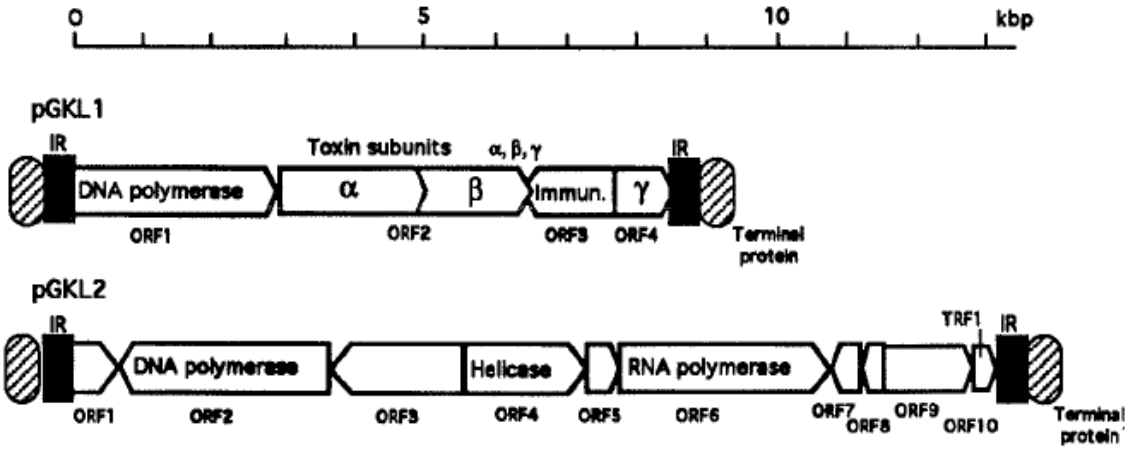

Figure 1.2 Structure of the two plasmids of *Kluyveromyces lactis* killer system. The two linear DNA plasmids, pGKL1 and pGKL2, of *K. lactis* are shown schematically. In pGKL1, ORF1 is thought to code for a DNA polymerase, ORF2 and 4 for three toxin subunits α, β and γ, and ORF3 for immunity (resistance to toxin) determinant; In pGKL2, ORF2, 4 and 6 are thought to code for a DNA polymerase, a helicase and an RNA polymerase, respectively; TGF1 has a role in plasmid replication. The terminal proteins are believed to be covalently attached to the DNA ends. IR, inverted terminal repeats (Fukuhara 1995).

1.2.3 *Williopsis mrakii* killer system

Williopsis mrakii produces two killer toxins encoded on chromosomal DNA, namely, HMK and K-500 (Magliani et al. 1997; Marquina et al. 2002). HMK, also referred to as HK-1, is a 10.7 kDa polypeptide rich in cysteine residues, and disulfide bonds seem to be the cause of its pH and thermostability; stable at pH 2-11, and at 100°C for 10 min (Magliani et al. 1997). Its killing activity is associated with the inhibition of β-1,3-glucan synthesis (Marquina et al. 2002). The second toxin, K-500, is a small polypeptide, 5.0 kDa, that exhibits killer activity against *Candida* species presumably by ion channel formation (Magliani et al. 1997).

1.2.4 *Ustilago maydis* killer system

The killer character in *Ustilago maydis* is encoded on dsRNA virus like particles (Koltin and Day 1976). *U. maydis* produces three different toxins, KP1, KP4 and KP6, with the last being the best studied (Finkler et al. 1992). KP6 killer toxin consists of two polypeptides, α (8.6 kDa) and β (9.1 kDa) (Magliani et al. 1997). Its mechanism of action is unknown. The α subunit is rich in cysteine and the disulfide bonds are crucial for its activity, presumably ion channel formation. Furthermore, the α subunit needs to interact with the cell wall so that the β subunits can cause the lethal effect (Steinlauf et al. 1988).

Table 1.1 Characteristics of the yeast killer toxins (Marquina et al. 2002).

Yeast species	Killer toxin	Subunits	Glyco-protein	Isoelectric point	Genetic basis	Primary receptor	Mechanism of killing	Application
Bullera sinensis	?	?	?	?	Chromosomal	?	?	?
Candida krusei	?	?	?	3.6–3.8	?	?	?	?
Candida glabrata	?	?	+	?	Chromosomal	?	Plasma membrane damage	?
Cryptococcus humicola	?	< 1 kDa	?	?	Chromosomal	?	?	?
Debaryomyces hansenii	?	23 kDa	?	?	Chromosomal	β-(1→6)-Glucan	?	?
Hanseniaspora uvarum	?	18 kDa	–	3.7–3.9	dsRNA	β-(1→6)-Glucan	?	?
Kluyveromyces fragilis	K6	42 kDa	?	?	?	?	?	?
Kluyveromyces lactis	?	α(97 kDa) β(31 kDa) γ(28 kDa)	?	?	dsDNA (pGKL1)	Chitin < ß2 >	Inhibition of cell cycle, G1 arrest	Avoid aerobic deterioration of silage
Kluyveromyces waltii	?	> 10 kDa	?	?	?	?	?	Control of *S. pombe* in wine making
Pichia acaciae	?	α(110 kDa) β(39 kDa) γ(38 kDa)	?	?	dsDNA (pPac1–2)	Chitin	?	Cell cycle arrest in G1, chitinase activity
Pichia anomala	?	83 kDa	?	?	?	?	?	Control of filamentous fungi in wood
Pichia farinosa	SMKT	α (6.3 kDa) β (7.7 kDa)	+	?	Chromosomal	?	Increase membrane permeability to ions	?
Pichia fermentans	?	?	?	3.8–4.2	?	?	?	?
Pichia inositovora	?	> 100 kDa	?	?	dsDNA (pPin1–3)	?	?	?
Pichia kluyveri	?	19 kDa	+	4.3	Chromosomal	?	Formation of ion channel	?
Pichia membranifaciens	?	18 kDa[a]	–	3.9[a]	Chromosomal[a]	β-(1→6)-Glucan	Formation of ion channel[a]	?
Saccharomyces cerevisiae	K1	α(9.5 kDa) β (9 kDa)	–	4.5	M_1-dsRNA	β-(1→6)-Glucan	Formation of ion channels, activation of K^+ channel	Avoid undesired contaminants in wine, beer, sake, etc. Genetics
S. cerevisiae	K2	$\alpha\beta$(21.5 kDa)	+	4.2–4.3	M_2-dsRNA	β-(1→6)-Glucan	Increase membrane permeability to ions	Wine fermentations
S. cerevisiae	KT28	α(10 kDa) β(11 kDa)	+	4.4	M_{28}-dsRNA	Manno-proteins	Entering into cell by endocytosis and inhibition of cell cycle, G2 arrest	?
Schwanniomyces occidentalis	?	α(7.4 kDa) β(4.9 kDa)	–	?	Chromosomal	Manno-proteins	Plasma membrane damage	?
Tilletiopsis albenscens	?	10 kDa	?	?	Chromosomal	?	?	?
Williopsis mrakii	HM-1	10.7 kDa	?	?	Chromosomal	β-(1→6)-, β-(1→3)-glucan	Inhibition of β-(1→3)-glucan synthesis	Silage, yogurt, taxonomy of *Nocardia*, Control of *C. albicans*
Williopsis saturnus	HYI	9,543 Da	–	5.8	Chromosomal	?	?	?
Zygosacch. bailii	KT412	10 kDa	–	4.1	dsRNA	Manno-proteins	?	?

1.3 Killer toxins applications

Numerous applications for killer yeasts have been proposed over the years (**Table 1.2**). Scientists have suggested their use as biocontrol agents against wood-decay and plant pathogenic fungi (Walker et al. 1995). Inhibition of spoilage organisms in the food industry is an important application that has been widely studied (Palpacelli et al. 1991), and special attention has been given within the wine industry due to the low pH required for the killing activity to occur (Heard and Fleet 1987; Jacobs et al. 1988; Tredoux et al. 1986). Santos looked at PMKT2 killer toxin produced by *Pichia membranifaciens* (Santos et al. 2009), and KP6 killer toxin produced by *Ustilago maydis* (Santos et al. 2011) and demonstrated the potential of both organisms as biocontrol agents to inhibit the growth of the spoilage yeast *Brettanomyces bruxellensis* in wine fermentations, due to the resistance that *Saccharomyces cerevisiae* exhibits towards these toxins. Also, he studied inhibition of *Botrytis cinerea* (a mold) by *P. membranifaciens* killer toxin in apples (Santos et al. 2004), and proposed its use as a biological control. The yeast *Williopsis saturnus* var. *saturnus*, which is incapable of fermenting lactose and/or galactose, was investigated for its potential as a biopreservative in cheese (Liu and Tsao 2009). Even inhibition of pathogenic bacteria like *Listeria monocytogenes* by food-borne yeasts has been investigated (Goerges et al. 2006).

Table 1.2 Potential applications of killer toxins.

Biotechnological field of application	Application
Biological control in agriculture	Antifungal activity against wood-decay and plant pathogenic fungi. Prevention of aerobic spoilage of silage.
Beverage fermentation	Avoid undesired contamination in wine, beer, sake, etc.
Cellular biology research on eukaryotic cells	Studies of biosynthesis, cellular processing and secretion of proteins.
Food technology	Food preservatives of natural origin.
Genetics	Selection of hybrids obtained by protoplast fusion. Fingerprinting of wine yeasts. Recombinant DNA technology (cloning vectors).
Medicine	Killer activities against pathogenic yeasts.
Taxonomy	Killer toxin sensitivity patterns may be indicative of phylogenetic affiliation.

Source (Marquina et al. 2002)

Moreover, researchers that have found killer activity against human pathogenic strains have encouraged the study of these organisms for the possible treatment of diseases (Banjara et al. 2016; Liu et al. 2015; Polonelli et al. 1986). The antifungal drug amphotericin B that is used to treat yeast infections (Vyas and Gupta 2006) induces permeability of ions through the membrane when used at high concentrations, which causes the antifungal antibiotic effect (Polonelli et al. 1991). This is similar to the mode of action of some killer toxins. Antifungal therapy against yeast infections can be

ineffective due to emerging resistance, and more treatment options are needed due to the increase in nosocomial infections among immunocompromised patients (Sobel 2006).

1.4 *Debaryomyces hansenii*

Debaryomyces hansenii (also identified in the literature as *Candida famata*, although it is probably a distinct but closely related species (Dmytruk and Sibirny 2012)) is a haploid yeast that reproduces by multilateral budding (Breuer and Harms 2006). It possesses many characteristics that make it an important yeast for biotechnological applications. The ability to synthesize and accumulate lipids, making it a so-called oleaginous yeast, has been studied by several scientists (Merdinger and Devine 1965; Tunblad-Johansson et al. 1987). The synthesis of D-arabitinol (Anderson and Harris 1963) and the capability to use inorganic ammonium as a nitrogen source (Breuer and Harms 2006; Yanai et al. 1994) are commercially attractive. *D. hansenii* produces large quantities of xylitol (Parajó et al. 1997; Roseiro et al. 1991), a sugar alcohol used in sugar-free foods due to its intense sweet taste (Sardesai and Waldshan 1991), diabetics' tolerance towards it, and anti-cavity properties (Isokangas et al. 1988). It also produces volatile compounds that are favorable for flavor development in dry-cured meat products (Andrade et al. 2010) and cheeses (Gori et al. 2012). Moreover, *D. hansenii* is an extremophile, one of the most halotolerant yeast species, growing in media containing up to 4M NaCl (Breuer and Harms 2006). It was originally isolated from saline environments (Kumar et al. 2012) and is among the most common yeasts found in cheese (Banjara et al. 2015) and in dry–cured meat products (Andrade et al. 2009). *D. hansenii* can also be found in environments with low water activity; hence its association with soil,

fruits, wine and beer (Breuer and Harms 2006; Kumar et al. 2012). *D. hansenii* exhibits considerable genetic diversity between strains (Corredor et al. 2003), reflected in the number of carbon substrates for which assimilation is denoted "variable" (**Table 1.3**). *D. hansenii* grows poorly in anaerobic conditions, contributing to the inability of some strains to ferment lactose, and leading to weak fermentation of galactose, sucrose, glucose, maltose and raffinose (Breuer and Harms 2006).

Table 1.3 Assimilation of substrates by *Debaryomyces hansenii*.

Glucose	+	*N*-Acetyl-D-glucosamine	V
Galactose	+	Methanol	–
L-Sorbose	V	Ethanol	+/W
Sucrose	+	Glycerol	+
Maltose	+	Erythritol	V
Cellobiose	+	Ribitol	+
Trehalose	+	Galacitol	V
Lactose	V	D-Mannitol	+
Melibiose	V	D-Glucitol	+/W
Raffinose	+	α-Methyl-D-glucoside	+
Melezitose	V	Salicin	+/W
Inulin	V	D-Gluconate	+/W
Soluble starch	V	DL-Lactate	V
D-Xylose	+	Succinate	+
L-Arabinose	+/W	Citrate	V
D-Arabinose	V	Inositol	–
D-Ribose	V	Hexadecan	V
L-Rhamnose	V	Nitrate	–
D-Glucosamine	V	Nitrite	V
2-Keto-D-gluconate	+	5-Keto-D-gluconate	V
Saccharate	–		

+ = positive, W = weak, V = variable, – = negative

Source (Breuer and Harms 2006)

1.4.1 *Debaryomyces hansenii* killer toxin production

Strains of *Debaryomyces hansenii* exhibit the killer phenotype and have been found to tolerate killer toxins from *Kluyveromyces lactis*, *Candida zeylanoides* and *Trichosporon cutaneum* (Fukuhara 1995; Nout et al. 1997). This suggests possible applications in food fermentations and preservation (Santos et al. 2002) and as a therapeutic agent (Banjara et al. 2016; Buzzini and Martini 2001). The genetic basis of the killer activity in *Debaryomyces hansenii* is unknown. Gunge reported the presence of three plasmids, pDHL1, pDHL2 and pDHL3, in *D. hansenii* (Gunge et al. 1993). Linear plasmid pDHL3 is essential for the replication of plasmids pDHL1 and pDHL2 (Fukuda et al. 1997). Plasmid pDHL1 is highly homologous to the plasmid pGKL1 present in *Kluyveromyces lactis* (**Table 1.4**), in which the linear dsDNA plasmid pGKL1 encodes a protein that is responsible for the killer phenotype (Fukuda et al. 1997; Gunge et al. 1993; Magliani et al. 1997). Santos and Marquina (Santos et al. 2002) were unable to eliminate the killer phenotype with various treatments used to eliminate plasmids (exposure to high temperatures, ultraviolet light and cycloheximide) and therefore concluded that the killer factor is chromosomally encoded. They reported that the killer toxin is a low molecular weight secreted protein, and identified 1→6-β-D-glucan as the cell wall binding site for this killer toxin. However, it is unknown if additional toxins may exist, as is the case of *Saccharomyces cerevisiae*, or how the killing mechanism of any of these toxins is achieved.

Corredor and colleagues reported high genome variability between strains of *Debaryomyces hansenii* based on chromosomal rearrangements and repeated and deleted sequences (Corredor et al. 2003). Banjara's work agrees with Corredor's findings, in so

far as *D. hansenii* isolates from cheese showed consistent, reproducible variation in killer toxin activity, suggesting genetic differences between them. Furthermore, his work confirmed reports from Marquina and colleagues (Marquina et al. 2001), where it was revealed that the killer activity is pH and temperature dependent with optimum conditions at 20°C and pH 4.5, and extended the findings to multiple *D. hansenii* strains (Banjara et al. 2016).

Mycocins produced by the cheese-derived yeast *Debaryomyces hansenii* represent an interesting area of research due to their ability to kill human fungal pathogens *Candida albicans* and *Candida tropicalis* in vitro. It is evident that more research is needed to understand the mechanisms by which these mycocins kill *Candida* species, so as to be able to use them as therapeutic agents for the possible treatment of fungal infections.

Table 1.4 Relatedness of gene sequences of the linear plasmids vs. *K. lactis* killer plasmids (Fukuhara 1995).

Host	Plasmid	pGKL1 gene probes					pGKL2 gene probes	
		ORF1 (DNA pol)	ORF2a (toxin α)	ORF2 (tox. α + β)	ORF3 (immun.)	ORF4 (toxin γ)	ORF2 (DNA pol)	ORF6 (RNA pol)
K. lactis	pKL2A	+ + +	+ + +	+ + +	+ + +	+ + +	–	–
	pKL2B	–	–	–	–	–	+ + +	+ + +
W. robert.	pWR1A	–	+	+	–	–	–	–
	pWR1B	–	–	–	–	–	+	+
D. hansen.	pDH1A	+	+	+	–	–	–	–
	pDH1B	–	–	–	–	–	+	+
P. etchel.	pPE1A	–	–	–	–	–	–	–
	pPE1B	–	–	–	–	–	–	+

Three pairs of linear plasmid DNAs were examined for the presence of homology with K. lactis killer DNA genes. Each DNA was hybridized with labeled individual gene probes from the K. lactis killer plasmids pGKL1 and 2. Data were taken from (Cong et al. 1994); + and – indicate presence and absence of hybrid signals, respectively. + + + corresponds to the intense signal of homologous hybridization at the same stringency condition. ORF2a is the N-terminal half of ORF2, coding for the toxin subunit α. *K. lactis = Kluyveromyces lactis, W. robert = Wingea robertsiae, D. hansen = Debaryomyces hansenii, P. etchel = Pichia etchellsii*.

1.5 *Candida* species as human pathogens

Candida species are commensal yeasts of the human skin, mouth, gut, and vagina (**Figure 1.3**) that usually cause no harm in healthy individuals. However, when the host system is altered, i.e. the immune system is suppressed, they take advantage and become pathogenic (see **Table 1.5** for risk factors). Infections caused by *Candida* yeasts (known as candidiasis) can range from mild cutaneous lesions and mucosal infections to severe systemic infections (bloodstream infections, i.e., candidemia, and invasive candidiasis, [IC]). *Candida albicans* is the most commonly isolated pathogenic species from infections, but there are other clinically important species such as *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis*, which are also frequently found in patients with fungal infections (Papon et al. 2013; Suhr 2015). *Candida* is the most common cause of hospital-acquired fungal infections (see **Table 1.7** for species breakdown) and is responsible for up to 40% mortality rate (Alonso-Valle et al. 2003). The incidence of yeast infections continues to increase every year (**Table 1.6**), probably due to the extensive use of antibiotics and an increasing population of immunosuppressed individuals. Additionally, drug-resistant species and strains are increasing in prevalence (Sobel 2006). Hence, biological controls constitute a valuable possibility for the treatment of these diseases.

Table 1.5 Major risk factors for invasive *Candida* infections (Perlroth et al. 2007).

Iatrogenic/Nosocomial Conditions*	Immunosuppression*
Colonization	Neutropenia
Broad spectrum antibiotics	Corticosteroids
Central venous catheter	HIV‡
Parenteral nutrition	Diabetes mellitus‡
Gastrointestinal or cardiac surgery	
Prolonged hospital stay†	
ICU stay	
Burns	
Premature neonate	

*Many iatrogenic/nosocomial conditions are accompanied by poorly characterized immune defects (e.g., burn injuries and surgery down modulate normal host defense mechanisms), as is diabetes mellitus.

‡HIV and diabetes mellitus predominantly predispose to mucocutaneous candidal infections, and diabetes is also a risk factor for disseminated disease; HIV is a cofactor for, but not an independent risk factor for, disseminated disease.

†Mean time to onset of disease in a recent, large, prospective study was day 22 of hospitalization.

Table 1.6 Trends in bloodstream infections caused by yeasts (Hazen 1995).

Year of Review	Summary and comments	Reference
1985	Studied only candidemia; frequency of species was *C. albicans* > *C. tropicalis* > *C. parapsilosis* > *C. glabrata* > *C. krusei* > other species	(Horn et al. 1985)
1986	Concerned with Virginia hospitals; found increase in *Candida* infections between 1978 and 1984 from 0.1 to 1.5 cases/10,000 patient discharges	(Morrison et al. 1986)
1989	Ranked nosocomial bloodstream infections; from 1984 to 1988, *Candida* species changed from eight to fourth most common agent of infection; genera of gram-negative bacilli are considered as individual categories	(Pfaller 1989)
1991	*Candida* species are fifth leading cause of bloodstream infection in 1989 (up from sixth in 1980) if gram-negative bacilli are considered one group	(Banerjee et al. 1991)
1991	Survey on fungemia for 1989 in one hospital in South Africa found that 2.1% of blood cultures contained yeasts; these included *C. albicans* (42%), *C. tropicalis* (26%), *C. parapsilosis* (20%), *C. glabrata* (7%), *Hansenula* spp. (2%), *C. guilliermondii* (1%), and *C. krusei* (1%).	(Badenhorst et al. 1991)
1992	Retrospective study in Indian teaching hospital; compared 5-yr periods 1980-1985 and 1986-1990; found 11-fold increase in candidemia; most common species isolated were *C. albicans*, *C. tropicalis*, *C. parapsilosis*, and *C. guilliermondii*	(Chakrabarti et al. 1992)
1992	Fungemia isolates in one hospital between 1984 and 1990; *C. albicans* > *C. tropicalis* = *C. glabrata* > *C. krusei* > *C. parapsilosis* = *C. guilliermondii*	(Meunier et al. 1992)

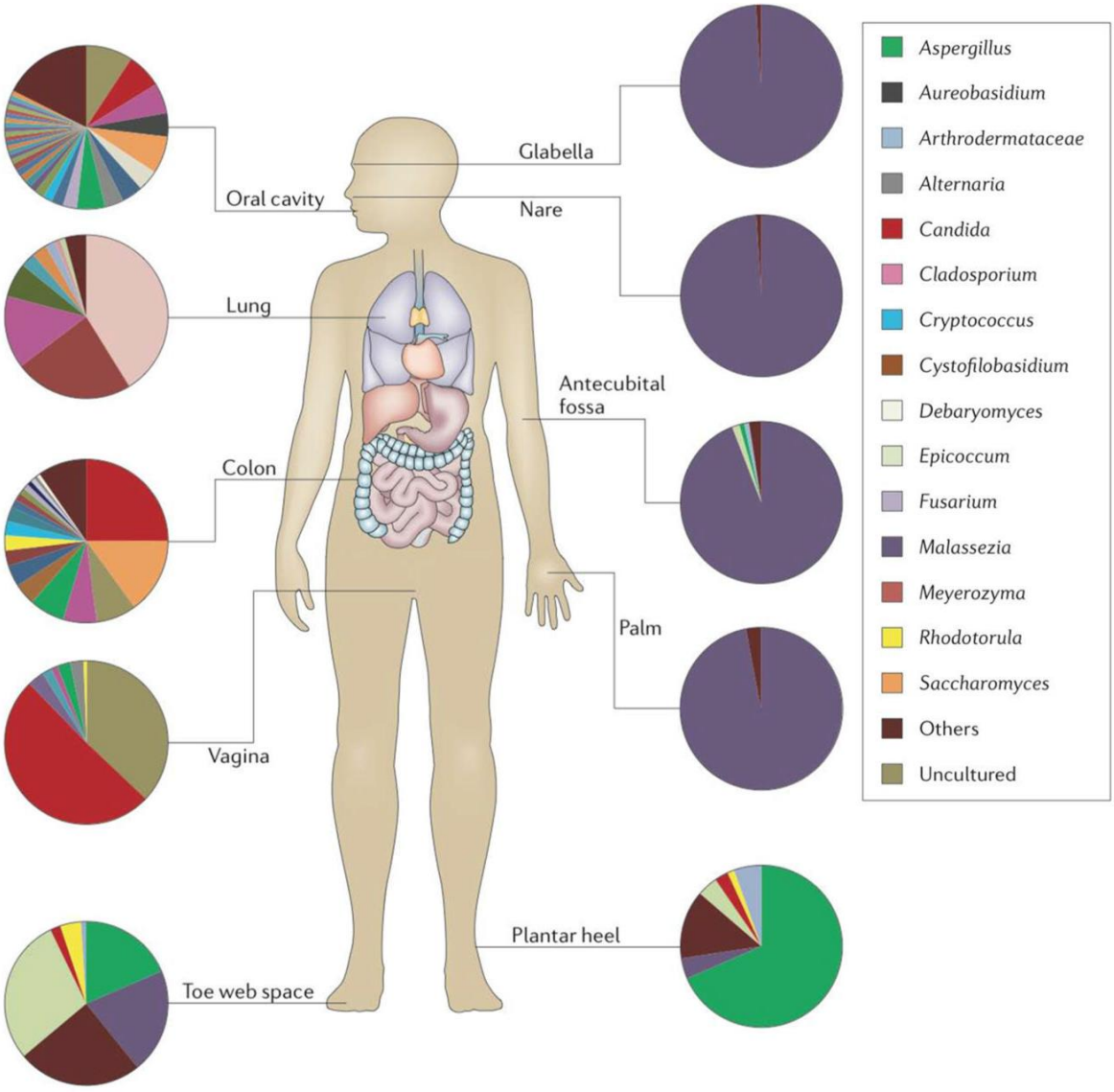

Figure 1.3 The human mycobiota (Underhill and Iliev 2014).

Table 1.7 Species breakdown of disseminated candidiasis (Perlroth et al. 2007).

Species	Percent of cases
Candida albicans	≈ 50%
Candida glabrata	≈ 15 – 25%
Candida parapsilosis	≈10 – 20%
Candida tropicalis	≈ 15%
*Candida krusei**	≈ <3%
Others	≈ <5%

*At cancer centers where significant fluconazole prophylaxis is used, *C. krusei* incidence may cause up to 10 – 15% of disseminated candidiasis.

1.5.1 *Candida albicans*

Candida albicans is the most commonly isolated species from patients with invasive infections, being responsible for 50% of *Candida* infections (Perlroth et al. 2007). It possesses a range of virulence factors (dimorphism, adhesins and invasins, formation of biofilms, secretion of hydrolytic enzymes) and fitness attributes (rapid stress, pH change and nutrient deficit adaptation) that account for its pathogenicity (Mayer et al. 2013). Dimorphism is the ability of the organism to change from a yeast form to hyphal form (Jacobsen et al. 2012). The yeast disseminates through the body and subsequently the formation of the hyphae expresses genes that encode for virulence factors (Mayer et al. 2013). Adhesins are proteins that facilitate the adherence of *C. albicans* to the cell surface, which is the first step towards infection (Garcia et al. 2011; Tsai et al. 2013). There are three different hydrolases produced by *C. albicans*: lipases, phospholipases and proteases (Williams et al. 2013). These hydrolytic enzymes help to

disseminate the infection (Wächtler et al. 2012) and in nutrient acquisition (Naglik et al. 2003). Additionally, biofilm formation is an important virulence factor. Biofilms are resistant to antimicrobials and antifungals, and the accumulation of yeast cells in medical equipment, e.g., catheters, present a serious risk for immunocompromised patients (Fanning and Mitchell 2012).

1.5.2 *Candida glabrata*

Candida glabrata is the second most common *Candida* species isolated from patients with candidemia in the US and Europe, accounting for 15 – 25% of *Candida* infections (Perlroth et al. 2007; Pfaller et al. 1998). It is closely related to *Saccharomyces cerevisiae*, unlike other *Candida* species (Roetzer et al. 2011). *C. glabrata* infections are more common in adults and are associated with azole prophylaxis, surgery and catheters (Krcmery and Barnes 2002). *C. glabrata* is resistant to azoles, which are the most commonly used antifungals due to their effectiveness against other *Candida* species. However, it is vulnerable to other antifungals like amphotericin B (Fidel et al. 1999).

1.5.3 *Candida tropicalis*

Candida tropicalis is responsible for 10 – 20% of *Candida* infections (Perlroth et al. 2007). It is among the most common non-*Candida albicans Candida* (NCAC) species isolated from infections (Negri et al. 2011) and it has been presenting resistance to fluconazole (Kothavade et al. 2010). Taxonomically speaking *C. tropicalis* is closely related to *C. albicans* (Negri et al. 2011). The rate of infections is correlated with several

risk factors including cancer, antibiotics, prolonged stay in hospitals, neutropenia and bone marrow transplants (Krcmery and Barnes 2002; Negri et al. 2011).

1.5.4 *Candida parapsilosis*

Candida parapsilosis accounts for 10 – 20% of *Candida* infections (Perlroth et al. 2007). Infections are a threat mostly to neonates and are related to hyperalimentation and body insertions (catheters) (Kojic and Darouiche 2004; Silva et al. 2012).

1.6 Anti-*Candida* activity by killer yeasts

Killer yeasts are known to have antimicrobial effects over sensitive species. *Williopsis mrakii*, *Hanseniaspora uvarum* and *Hansenula anomala* are a few of the yeast that exhibit anti-candida activity (Hodgson et al. 1995; Schmitt et al. 1997). More recently, killer toxin produced by marine-derived yeast *Wickerhamomyces anomalus* has been found to produce killer toxins against *C. albicans* and *C. tropicalis* at pH 3.5 and 16°C (Guo et al. 2013), and food-derived yeast *Debaryomyces hansenii* has been found to produce killer toxins against *C. albicans* and *C. tropicalis* at pH 4.5 and 20°C (Banjara et al. 2016). Killer toxins depend on pH and temperature for their action, and these differ between species; being prevalent low pH and temperature (~20°C) for the killing activity.

Most of the studies in killer yeasts have been performed *in vitro*, especially those against *Candida* species. Killing *in vivo* has not yet been reported. The human body temperature is 37°C and due to the low temperature needed for the killing activity of mycocins to occur, the therapeutic use of these toxins *in vivo* presents a challenge. To be able to use killer toxins as antifungals, the toxin would need to overcome several

obstacles, including being active at 37°C, surviving the acidity of the stomach and finally reaching the lower gastrointestinal tract, which is where most of the drugs are absorbed (Martinez and Amidon 2002).

Nonetheless, this presents itself as an important research field. The extensive use of antibiotics, the increase in the number of immunocompromised patients and the amount of pathogens becoming resistant to antifungals are some of the reasons why yeast infections are continuing to increase every year (Hazen 1995; Pfaller 1989; Sobel 2006).

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Chapter 2

Effect of *Debaryomyces hansenii* killer toxin against pathogenic bloodstream *Candida* isolates.

2.1 Abstract

Candida species are commensal organisms (part of the normal human microbiota), but when the host immune system is disrupted they can become pathogenic. Antifungal therapy against yeast infections can be ineffective due to emerging resistance correlated with their prolonged use, and more treatment options are needed. Yeast production of toxic proteins or glycoproteins, i.e. killer toxins or mycocins, was first discovered in *Saccharomyces cerevisiae*, and it has been observed in more than 90 species. One of the applications of killer toxins is in the field of biotechnology, as is the case for *Debaryomyces hansenii*, which was found to produce toxins against common human fungal pathogens *Candida albicans* (SC5314) and *Candida tropicalis* (NRRL-10985). However, the extent of yeast species and strains susceptible to *D. hansenii's* toxin(s), and the nature of this susceptibility, remain unknown. To expand the knowledge on the extent of pathogenic *Candida* species susceptible to *Debaryomyces hansenii* killer toxin, nineteen *D. hansenii* strains were assayed for killer activity against nine bloodstream isolates from six pathogenic yeast species. No strain had any effect on bloodstream *Candida albicans*, *Candida parapsilosis* or *Candida orthopsilosis* under the conditions tested. The single *Wickerhamomyces anomalus* strain was susceptible to the same *D. hansenii* strains that killed *Candida tropicalis* NRRL-10985, and *Candida glabrata* presented an unusual, but very interesting susceptibility pattern. Additionally, *D. hansenii* strains were assayed for killing activity against each other, with no such activity detected. Finally, protein biosynthesis was inhibited with cycloheximide so as to investigate whether the toxin was coded cytoplasmically or chromosomally. Killer toxin assay on samples treated with cycloheximide suggests that the toxin is encoded in the

chromosome (instead of cytoplasmically) and that there exist numerous differences between *D. hansenii* strains that may account for the difference in toxicity.

2.2 Introduction

Candida species are commensal yeasts of the human skin, mouth, gut and vagina, usually causing no harm in healthy individuals. However, when the host system is altered, i.e. the immune system is suppressed, they take advantage and become pathogenic. Infections caused by *Candida* yeasts (known as candidiasis) can range from mild cutaneous lesions and mucosal infections to severe systemic infections (bloodstream infections, i.e., candidemia, and invasive candidiasis, [IC]). *Candida albicans* is the most commonly isolated pathogenic species from infections, but there are other clinically important species such as *C. glabrata*, *C. parapsilosis*, *C. krusei* and *C. tropicalis,* which are also frequently found in patients with fungal infections (Papon et al. 2013; Suhr 2015). The incidence of yeast infections continues to increase every year, probably due to the extensive use of antibiotics and an increasing population of immunosuppressed individuals. Additionally, drug-resistant species and strains are increasing in prevalence (Sobel 2006). Hence, biological controls constitute a valuable possibility for the treatment of these diseases.

Makower and Bevan first discovered the killer character in *Saccharomyces cerevisiae* in 1963 (Woods and Bevan 1968). Killer yeasts produce toxic proteins or glycoproteins (also called mycocins), and this characteristic has been found in more than 90 yeast species (Buzzini and Martini 2001; Young and Yagiu 1978). Killer yeasts are able to kill sensitive yeast species and they possess intrinsic immunity to the action of

their own mycocin (Schmitt and Breinig 2002). The genetic basis of the killer phenotype varies; it can be chromosomal or cytoplasmic (viral dsRNA or linear dsDNA plasmids). Killer yeasts can produce more than one toxin, as is the case for the best studied organism *Saccharomyces cerevisiae*, which produces 5 different toxins: K1, K2 and K28, which are cytoplasmically encoded (viral dsRNA) and KHR and KHS which are encoded on chromosomal DNA (Magliani et al. 1997). The mechanism of action by which these mycocins kill sensitive species varies. It has been shown that they can do so by inhibiting DNA replication, inducing changes in membrane permeability (pore formation) or by cell cycle arrest in G1 phase, among others (Liu et al. 2015).

Debaryomyces hansenii is a haploid yeast that reproduces primarily by budding. It can be found in environments with low water activity and it is one of the most halotolerant yeast species (up to 25% NaCl); hence its association with cheese, meat, soil, wine and beer (Breuer and Harms 2006; Kumar et al. 2012). *D. hansenii* exhibits the killer phenotype and it has been found to produce and tolerate killer toxins (Banjara et al. 2016; Breuer and Harms 2006; Santos et al. 2002). This characteristic suggests possible applications in food fermentations and preservation (Santos et al. 2002) and as a therapeutic agent (Banjara et al. 2016; Buzzini and Martini 2001).

The genetic basis of the killer activity in *Debaryomyces hansenii* is unknown. Gunge reported the presence of plasmids, pDHL, in *D. hansenii*, but studies show that the killer activity is not associated with this plasmid; while in *Kluyveromyces lactis*, a linear dsDNA plasmid encodes the killer phenotype (Fukuda et al. 1997; Gunge et al. 1993). Santos and Marquina (Santos et al. 2002) reported that the killer toxin is a low molecular weight secreted protein encoded in chromosomal DNA, and identified 1→6-β-D-glucan

as the cell wall binding site for this killer toxin. However, it is unknown if additional toxins may exist, as is the case of *Saccharomyces cerevisiae*, or how the killing mechanism of these toxins is achieved.

Corredor and colleagues reported high genome variability between strains of *Debaryomyces hansenii* based on chromosomal rearrangements and repeated and deleted sequences (Corredor et al. 2003). Banjara's work agrees with Corredor's findings, in so far as *D. hansenii* isolates from cheese showed consistent, reproducible variation in killer toxin activity, suggesting genetic differences between them. Furthermore, his work confirmed reports from Marquina and colleagues (Marquina et al. 2001), where it was revealed that the killer activity is pH and temperature dependent, with optimum conditions at 20°C and pH 4.5, and extended the findings to multiple *D. hansenii* strains (Banjara et al. 2016).

Mycocins produced by the cheese-derived yeast, *Debaryomyces hansenii*, represent an interesting research field due to their ease of isolation and ability to kill human fungal pathogens *Candida albicans* and *Candida tropicalis in vitro*. It is evident that more research is needed to understand the breadth of action of these mycocins against *Candida* species, so as to be able to use them as antifungal therapeutic agents for the possible treatment of these infections. In this study, we intend to broaden the knowledge on the diversity of infectious species and strains affected by this toxin, executing *in vitro* assays using previously isolated pathogenic *Candida* from bloodstream infections.

2.3 Materials and Methods

Fungal Strains, growth media and culture conditions

YEPD rich media (1% yeast extract, 2% peptone, 2% dextrose) was used to grow nineteen strains of *Debaryomyces hansenii* (**Table 2.1**), previously isolated from a variety of cheeses (Banjara et al. 2016), at room temperature. Strains were designated "killer" or "non-killer" based on killer activity against *Candida albicans* SC5314 and *C. tropicalis* NRRL 10985 (Banjara et al. 2016). Human pathogenic yeast strains were grown in YEPD at a temperature of 37°C (**Table 2.2**). All yeasts were maintained on YEPD slants at room temperature, and at -86°C in 35% glycerol.

Table 2.1 *Debaryomyces hansenii* strains and their source of isolation (Banjara et al. 2016).

Strain ID	Cheese	Killer phenotype
Dhans-10	Queso Authentico	+
Dhans-34	Italian Bel Pase	+
Dhans-45	Belgioloso Romano	+
Dhans-46	Wisconsin Roth Kase	+
Dhans-65	Landana 1000 days (Gouda)	+
Dhans-68	Queso Authentico	–
Dhans-72	Hennings Colby	–
Dhans-75	Italian Belpase	–
Dhans-76	Cheddar (Black Creek Smooth and Creamy)	–
Dhans-201	Bavarian Red (Rougetta)	+
Dhans-220	Italian Bel Paese (Galbani)	+
Dhans-237	Italian Bel Paese	+
Dhans-242	Parmesan (Reggiano)	+
Dhans-246	Raclette (Grand Cru)	+
Dhans-255	Provolone Cheese (Dilusso's Wisconsin)	+
Dhans-262	Ricotta	+
Dhans-265	Wensleydale cheese (Coombe castle)	+

Dhans-274	Blue cheese (Roth Kase Minis)	+
Dhans-276	Blue cheese (Roth Kase Minis)	+
CBS 767	Sequenced strain (http://genolevures.org/deha.html)	

Table 2.2 Pathogenic strains and source of isolation (Suhr 2015).

Pathogenic species	Strain ID	Source of Isolation
Candida albicans	SC5314	Laboratory strain
Candida albicans	B1783	Blood
Candida albicans	B4549	Blood
Candida tropicalis	NRRL-10985	Laboratory Strain
Candida parapsilosis	B0595	Blood
Candida parapsilosis	B9860	Blood
Candida parapsilosis	B9832	Blood
Candida parapsilosis	B4075	Blood
Candida orthopsilosis	B4791	Blood
Candida glabrata	B9872	Blood
Wickerhamomyces anomalus	B1448	Blood

Killer toxin activity assays

Killer toxin activity of *Debaryomyces hansenii* killer strains was evaluated against pathogenic species and strains (see **Table 2.2** for pathogenic strains), and against non-killer *D. hansenii* strains. Killer toxin activity was determined using the streak-plate agar diffusion bioassay (Hodgson et al. 1995; Rosini 1983). YEPD-methylene blue agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 0.003% methylene blue and 10mM sodium citrate) was prepared, and pH was adjusted to 4.5 with HCl. The media was autoclaved and cooled to 45°C. Subsequently, it was seeded with pathogenic or *D. hansenii* strains to a final density of 10^5 cells ml^{-1}. Ultimately, 15 ml were poured into

each plate and after 5 hours, colonies of *D. hansenii* were streaked on the surface of the agar. Plates were incubated at 20°C and examined daily. Toxin production, a killer positive reaction, was determined visually by the presence of an inhibition zone (see **Figure 2.1**), whereas the absence of the inhibition zone indicated lack of killer activity (Hernández et al. 2008).

Cold shock killer toxin assay

Killer toxin activity of *Debaryomyces hansenii* strains was evaluated against pathogenic species by streak-plate agar diffusion bioassay (Hodgson et al. 1995; Rosini 1983). YEPD-methylene blue agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 0.003% methylene blue and 10mM sodium citrate) was prepared, and pH was adjusted to 4.5 with HCl. The media was autoclaved and cooled to 45°C. Subsequently, it was seeded with pathogenic strains to a final density of 10^5 cells ml^{-1}. 15 ml were poured into each plate and after 5 hours, colonies of *D. hansenii* were streaked on the surface of the agar. Plates were incubated at 10°C for 24 hours and then placed at 20°C. Indication of toxin production was evaluated as previously described.

Plasmid curing

Debaryomyces hansenii killer strains were grown at room temperature in YEPD media (1% yeast extract, 2% peptone and 2% dextrose) containing cycloheximide at concentrations of 1 mg ml^{-1} and 10 mg ml^{-1}. Alternatively, YEPD-methylene blue agar (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 0.003% methylene blue and 10mM sodium citrate) was prepared and pH was adjusted to 4.5 with HCl (Santos et al.

2002). The media was autoclaved and cooled to 45°C. Subsequently, it was seeded with *Candida albicans* SC5314 and *Candida tropicalis* NRRL-10985 to a final density of 10^5 cells ml^{-1} and cycloheximide at concentrations of 1 mg ml^{-1} and 10 mg ml^{-1} was added. Ultimately, 15 ml were poured into each plate and after 5 hours, colonies of *D. hansenii* (Dhans-237, Dhans-276 and CBS 767) were streaked on the surface of the agar and incubated at 20°C. Indication of toxin production was evaluated as previously described.

2.4 Results and Discussion

From nineteen cheese-derived *Debaryomyces hansenii* strains, Banjara and colleagues (2016) reported fifteen strains with killing activity and four strains which consistently do not exhibit the killer phenotype (**Table 2.1**). However, they only tested killer activity against a single strain each of *Candida albicans* and *Candida tropicalis.* To expand the knowledge on the extent of pathogenic *Candida* species susceptible to *Debaryomyces hansenii* killer toxin, we reproduced Banjara's assays against *Candida albicans* SC5314 and *Candida tropicalis* NRRL-10985, and additionally tested nine pathogenic *Candida* strains previously isolated from small bowel transplant patients with bloodstream infections (Suhr 2015). Our results mostly confirmed Banjara's on *C. albicans* SC5314 and *C. tropicalis* NRRL-10985, with *C. tropicalis* more sensitive than *C. albicans*; however, strain Dhans-255 failed to kill *C. tropicalis* in our studies. Interestingly, Dhans-255 is morphologically distinct, having a rough surface when grown on solid culture medium, whereas the rest of the *D. hansenii* strains appear smooth. No *D. hansenii* strain exhibited killing activity against bloodstream isolates of *Candida*

albicans (B1783 and B4549), *Candida parapsilosis* (B9860, B0595, B9832 and B4075) and *Candida orthopsilosis* B4791. However, the bloodstream isolate of *Wickerhamomyces anomalus* (B1448) displayed the same sensitivity profile as *Candida tropicalis* NRRL-10985 (**Table 2.3** and **Table 2.4**).

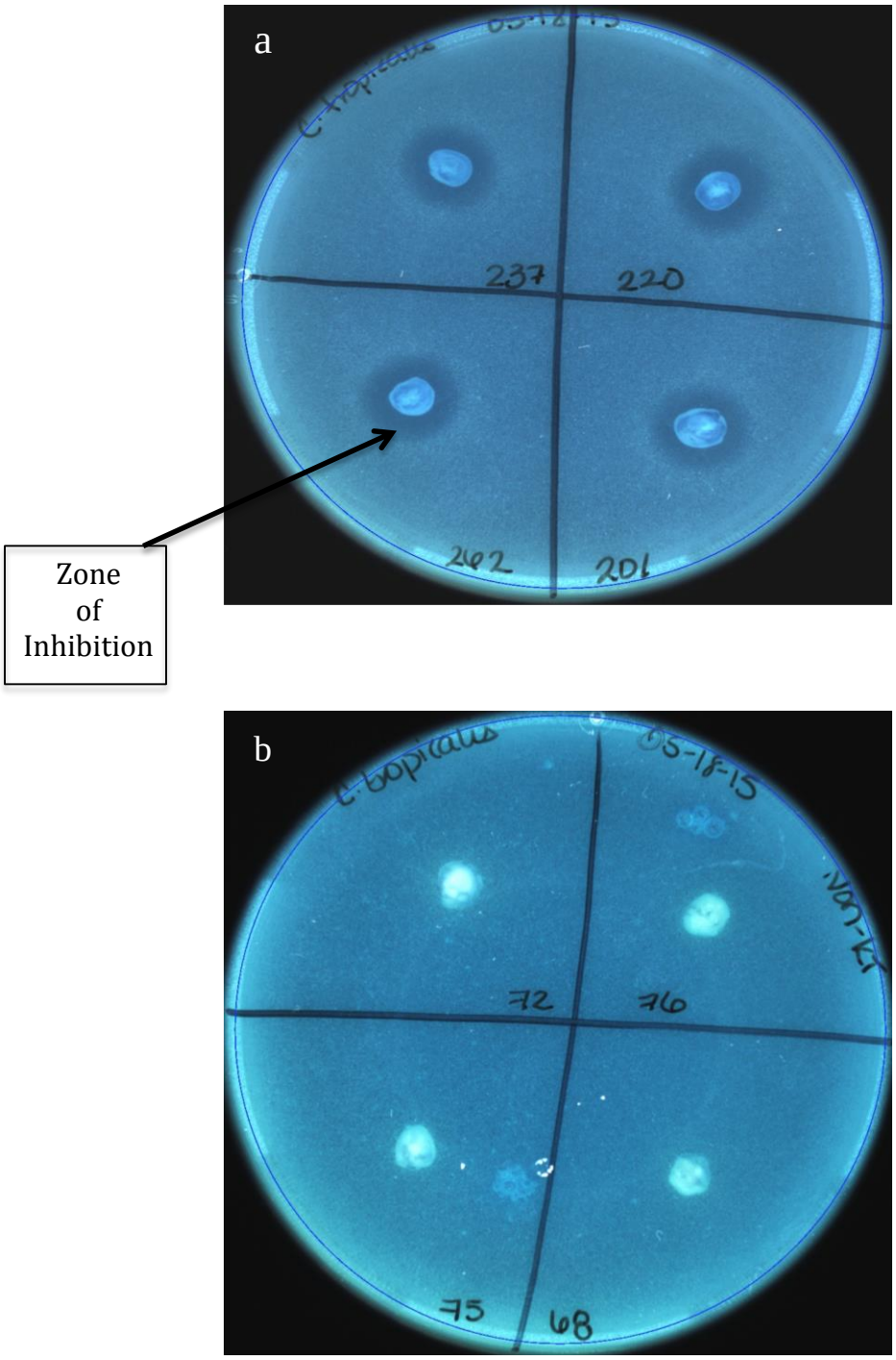

Figure 2.1 Killer assay. YPD methylene blue plate agar seeded with *Candida tropicalis* NRRL-10985 and then streaked with killer and non-killer strains of *Debaryomyces hansenii*. Killer strains (a) show a zone of inhibition after 24 hours of incubation at 20°C, as opposed to non-killer strains (b) where no inhibition zone is observed.

Table 2.3 Killer toxin activity against pathogenic species at 20°C.

Strain	***Candida albicans***			***Candida parapsilosis***				***C. ortho***	***C. glab***	***C. trop***	***W. anom***
	SC5314	B1783	B4549	B9860	B0595	B9832	B4075	B4791	B9872	NRRL-10985	B1448
Dhans-10	+	–	–	–	–	–	–	–	–	+	+
Dhans-34	+	–	–	–	–	–	–	–	–	+	+
Dhans-45	+	–	–	–	–	–	–	–	–	+	+
Dhans-46	+	–	–	–	–	–	–	–	–	+	+
Dhans-65	+	–	–	–	–	–	–	–	–	+	+
Dhans-68	–	–	–	–	–	–	–	–	–	–	–
Dhans-72	–	–	–	–	–	–	–	–	–	–	–
Dhans-75	–	–	–	–	–	–	–	–	–	–	–
Dhans-76	–	–	–	–	–	–	–	–	–	–	–
Dhans-201	+	–	–	–	–	–	–	–	–	+	+
Dhans-220	+	–	–	–	–	–	–	–	–	+	+
Dhans-237	+	–	–	–	–	–	–	–	–	+	+
Dhans-242	+	–	–	–	–	–	–	–	–	+	+
Dhans-246	+	–	–	–	–	–	–	–	–	+	+
Dhans-255	+	–	–	–	–	–	–	–	–	–	–
Dhans-262	+	–	–	–	–	–	–	–	–	+	+
Dhans-265	+	–	–	–	–	–	–	–	–	+	+
Dhans-274	+	–	–	–	–	–	–	–	–	+	+
Dhans-276	+	–	–	–	–	–	–	–	–	+	+

C. ortho = Candida orthopsilosis, C. glab = Candida glabrata, C. trop = Candida tropicalis, W. anom = Wickerhamomyces anomalus

+ = Killer positive, presence of an inhibition zone

– = Killer negative, no inhibition zone observed

Table 2.4 Killer toxin activity against pathogenic species after 24 hour cold shock.

Strain	***Candida albicans***			***Candida parapsilosis***				***C. ortho***	***C. glab***	***C. trop***	***W. anom***
	SC5314	B1783	B4549	B9860	B0595	B9832	B4075	B4791	B9872	NRRL-10985	B1448
Dhans-10	+	–	–	–	–	–	–	–	–	+	+
Dhans-34	+	–	–	–	–	–	–	–	v	+	+
Dhans-45	+	–	–	–	–	–	–	–	+	+	+
Dhans-46	+	–	–	–	–	–	–	–	–	+	+
Dhans-65	+	–	–	–	–	–	–	–	+	+	+
Dhans-68	–	–	–	–	–	–	–	–	–	–	–
Dhans-72	–	–	–	–	–	–	–	–	–	–	–
Dhans-75	–	–	–	–	–	–	–	–	–	–	–
Dhans-76	–	–	–	–	–	–	–	–	–	–	–
Dhans-201	+	–	–	–	–	–	–	–	+	+	+
Dhans-220	+	–	–	–	–	–	–	–	–	+	+
Dhans-237	+	–	–	–	–	–	–	–	+	+	+
Dhans-242	+	–	–	–	–	–	–	–	+	+	+
Dhans-246	+	–	–	–	–	–	–	–	+	+	+
Dhans-255	+	–	–	–	–	–	–	–	–	–	–
Dhans-262	+	–	–	–	–	–	–	–	+	+	+
Dhans-265	+	–	–	–	–	–	–	–	+	+	+
Dhans-274	+	–	–	–	–	–	–	–	+	+	+
Dhans-276	+	–	–	–	–	–	–	–	–	+	+

C. ortho = Candida orthopsilosis, *C. glab = Candida glabrata*, *C. trop = Candida tropicalis*, *W. anom = Wickerhamomyces anomalus*

+ = Killer positive, presence of an inhibition zone, – = Killer negative, no inhibition zone observed

v = Killer activity variable

Candida glabrata, the second most common *Candida* species isolated from patients with candidemia (Pfaller et al. 1998), presented an interesting profile. No *D. hansenii* strains exhibited killer activity against *C. glabrata* when assayed at a uniform 20°C. Due to a faulty thermostat in our incubator, we incubated *the D. hansenii* vs. *C. glabrata* interaction at 10°C for 24 hours before we realized the thermostat flaw and shifted the plates to 20°C for the remaining incubation. Nine strains exhibited killing activity against this pathogen after five days (**Figure 2.2**, **Table 2.4**). However, when trying to replicate this experiment we obtained a third different profile, which included strain Dhans-34 presenting mycocin activity contrary to the previous assay. Cold shock did not alter the killer profile against any other strain from that observed at 20°C, although we observed clearer and larger zones of killing in assays that had undergone a cold shock. *D. hansenii* favors lower temperatures than the pathogenic species (Breuer and Harms 2006), and the cold shock may have disproportionately slowed *Candida* and *Wickerhamomyces* growth relative to *D. hansenii*, allowing more time for killer toxin production. *C. glabrata* is a distant relative to the other yeasts in this study, being closely related to *Saccharomyces cerevisiae* (Roetzer et al. 2011). Further study would be necessary to posit a mechanism for the observed cold shock effect, and to determine whether it would have any relevance *in vivo*.

Nevertheless, this presents itself as an interesting research field, due to the similarities that *Candida glabrata* possesses with *Saccharomyces cerevisiae* (Roetzer et al. 2011). Considering that *D. hansenii* is highly influenced by higher salt concentrations, conducting this assay at higher NaCl concentrations would give us a better understanding of the killing phenotype.

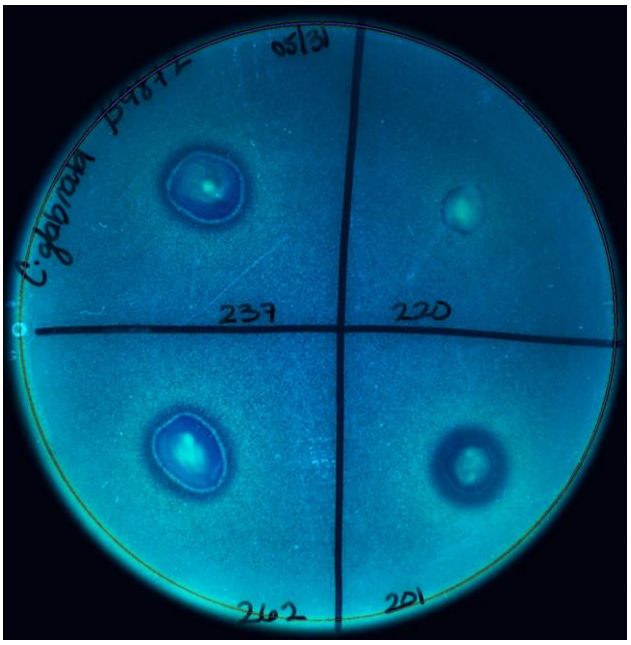

Figure 2.2 Cold shock killer assay. YPD methylene blue plate agar seeded with bloodstream isolate of *Candida glabrata* B9872 and then streaked with strains of *Debaryomyces hansenii*. Killer strains (Dhans-237, Dhans-262 and Dhans-201) show a zone of inhibition after 24 hours of incubation at 10°C followed 20°C incubation for seven days, as opposed to Dhans-220 where no inhibition zone is observed.

Killer yeasts, as a self-protection mechanism, are immune to the action of the toxin they produce (Bussey 1991; Schmitt and Breinig 2006). A killer toxin assay performed between the *D. hansenii* strains that exhibited the killer phenotype and the ones that did not show killing activity revealed that even strains lacking killer activity in any of our assays possess immunity to the action of the toxins from their own species (see **Table 2.5**). In *Saccharomyces cerevisiae*, in which the killer phenomenon was first described, a wide range of phenotypes exists: killer strains which both kill and are resistant to killer toxin (K+R+), susceptible strains which neither kill nor are resistant (K-R-), neutral strains which do not kill but are resistant to mycocins (K-R+), and, rarely,

"suicidal" strains which kill but possess reduced immunity to their own mycocins (K+ R-) (Polonelli et al. 1991). Thus far we have only observed killer and neutral *D. hansenii* strains; increased sampling may reveal further diversity.

Table 2.5 Killer toxin activity of *Debaryomyces hansenii* killer strains against *D. hansenii* non-killer strains

Strains	Dhans-68	Dhans-72	Dhans-75	Dhans-76
Dhans-10	–	–	–	–
Dhans-34	–	–	–	–
Dhans-45	–	–	–	–
Dhans-46	–	–	–	–
Dhans-65	–	–	–	–
Dhans-201	–	–	–	–
Dhans-220	–	–	–	–
Dhans-237	–	–	–	–
Dhans-242	–	–	–	–
Dhans-246	–	–	–	–
Dhans-255	–	–	–	–
Dhans-262	–	–	–	–
Dhans-265	–	–	–	–
Dhans-274	–	–	–	–
Dhans-276	–	–	–	–

– = Killer negative, no inhibition zone observed

Debaryomyces hansenii possesses three plasmids: pDHL1 pDHL2 and pDHL3 (Gunge et al. 1993). Plasmid pDHL1 is highly homologous to the plasmid pGKL1 present in *Kluyveromyces lactis*, which encodes a protein that is the source of the killer phenotype (Fukuda et al. 1997). Killer yeasts can be cured of plasmid-encoded killer phenotype by a series of methods that include, but are not limited to, exposure to high temperatures, ultraviolet light and cycloheximide (Santos et al. 2002). To determine

whether the killer activity of any *D. hansenii* strain was encoded on a plasmid, strains were grown with cycloheximide to eliminate cytoplasmic plasmids. Attempts to grow *D. hansenii* in a media containing cycloheximide were unsuccessful as a result of protein biosynthesis inhibition caused by cycloheximide. Alternative treatment in effort to cure *Debaryomyces hansenii*'s plasmid with cycloheximide was ineffective (see **Figure 2.3**). Our results concur with those of Buzzini and Martini (2000) and Santos (2002)(Buzzini and Martini 2000; Santos et al. 2002), in which treated *Debaryomyces hansenii* showed no reduced activity during killer activity assays on methylene blue plates seeded with a susceptible species (e.g. *Candida tropicalis*). Killer strains retaining the killer phenotype after treatment suggest that the killer phenotype is not associated with the presence of a plasmid. Further tests could be performed to ensure absence of plasmids in treated cells; however, all available evidence to date supports the hypothesis of a chromosomal location for *D. hansenii* mycocins-encoding gene(s).

Significantly, studies on killer and susceptible yeasts show that strain sensitivity can be influenced, among other things, by the nature of the cell wall and physiological niche (Hodgson et al. 1995). Considering *Candida* as an opportunistic pathogen, but also a commensal organism of the human microbiota, virulence and other physiological parameters differ between isolates. The correlation, if any, between virulence in a human host and resistance or susceptibility to killer toxins remains unknown. Recent bloodstream isolates such as *C. albicans* B1783 and B4549 might be expected to be more aggressive and robust than *C. albicans* SC5314, which has been subcultured for 30+ years since its original isolation from a patient with disseminated candidiasis. The inability of any of our *D. hansenii* killer strains to kill the bloodstream *C. albicans* (and

the ability to kill SC5314) demonstrate clearly the need to screen a broad range of potentially susceptible isolates as well as killer isolates. Candidemia is a serious life-threatening infection that can affect the brain, heart and bones (Pappas 2006), against which new therapeutic options are desperately needed. Further sampling may reveal strains or combinations of strains of killer yeasts with greater efficacy against pathogenic *Candida*, while purification of the mycocins(s) from *D. hansenii* and characterization of the mode of action may yield a usable antimycotic agent.

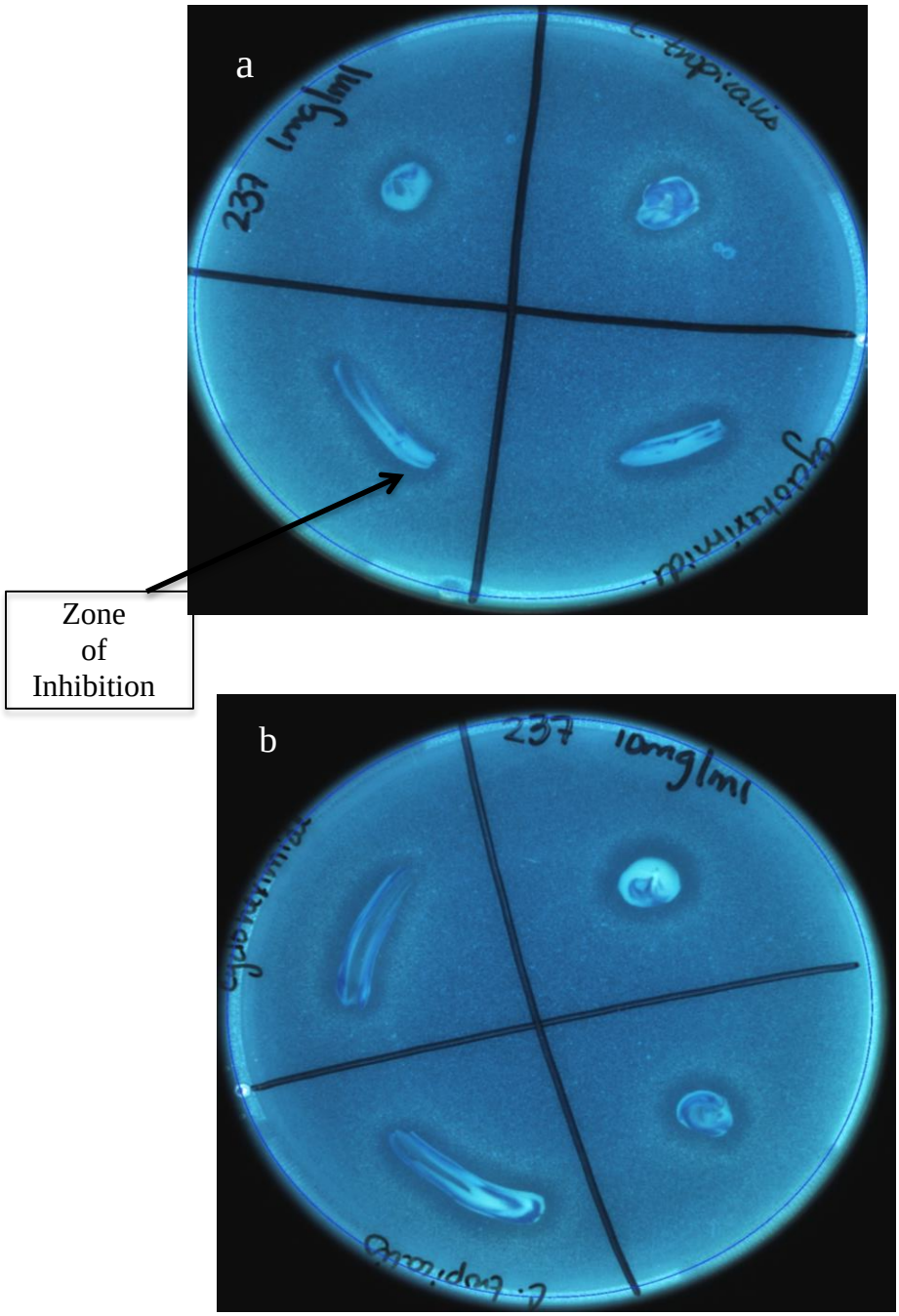

Figure 2.3 Plasmid-curing killer assay. YPD methylene blue plate agar seeded with *Candida tropicalis* NRRL-10985 and cycloheximide at concentrations of 1 mg ml^{-1} (a) and 10 mg ml^{-1} (b), subsequently streaked with killer strains of *Debaryomyces hansenii* show a zone of inhibition after 24 hours of incubation at 20°C.

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Chapter 3

Towards understanding the genetic basis of *Debaryomyces hansenii* killer toxin

3.1 Abstract

Yeasts can produce toxic proteins or glycoproteins, i.e. “mycocins” or “killer toxins”, which are able to kill sensitive yeast species. The production of mycocins, first discovered in *Saccharomyces cerevisiae*, is a widespread phenomenon among various yeast genera. *Debaryomyces hansenii* is one of the most halotolerant yeast species, hence its association with preserved and fermented foods. Strains of *D. hansenii* have been shown to kill potentially pathogenic yeasts, including strains of *Candida albicans*, *Candida glabrata*, *Candida tropicalis,* and *Wickerhamomyces anomalus* at optimum conditions of 20°C and pH 4.5. The genetic basis and mechanism of action of this toxin(s) are unknown. Cycloheximide treatment of *D. hansenii* prior to or during killer activity assays on methylene blue plates seeded with *Candida tropicalis* does not reduce mycocin activity, suggesting that the toxin is not cytoplasmically-encoded, as are several characterized yeast killer toxins, but chromosomal. To understand the nature of these toxins, 2x250 paired end Illumina sequencing, averaging 100x coverage (range: 48x - 145x), was performed on 7 killer and 3 non-killer strains of *Debaryomyces hansenii* previously isolated from different types of cheese. Numerous differences between *D. hansenii* strains were identified that may account for the difference in toxicity. Genome analysis detected 48 DNA regions, ranging from 31-3434 bp, unique to all sequenced killer strains and missing from non-killer strains, and 32 regions present in all non-killers and missing from killer strains. Five different regions have been chosen for their possible killer toxin role, including predicted members of the ABC-ATPase and major facilitator superfamilies, cytochrome P450s, and interestingly, a multidrug resistance protein.

Primers were designed and construction of a mutant has begun so as to be one step closer to understanding the relationship between these regions and the killer toxin production.

3.2 Introduction

The killer phenomenon among yeasts is fairly common, having been reported in more than 90 species, across more than 20 genera (Walker et al. 1995; Young and Yagiu 1978). Killer yeasts produce toxic proteins that are able to kill susceptible species. The genetic basis of these toxins can be encoded on chromosomal genes or in the cytoplasm as dsRNA virus-like particles or linear dsDNA plasmids (Magliani et al. 1997). The modes of action to achieve the lethal effect vary and, usually, as a self defense mechanism they possess intrinsic immunity to the action of their own mycocin (Schmitt and Breinig 2002). However, there exist situations where mycocin concentration prevails and this immunity is overcome; so called suicidal mutants (K+ R–) (Polonelli et al. 1991). Young stated that while all killer systems vary, they have a proteinaceous compound crucial to the killer activity that is common among them, and yeasts from the same species can produce various toxins with distinctive modes of action (Young and Yagiu 1978).

The first requirement to kill a susceptible organism is commonly a cell wall receptor on the sensitive cell that interacts with the killer toxin, usually chitin, β-glucans or mannoproteins, and suggestions exist about the existence of a second receptor on the plasma membrane that facilitates absorption (Magliani et al. 1997; Schmitt and Breinig 2006). Subsequently, they can achieve the lethal effect by different biological pathways.

K1 and K2 toxin produced by *Saccharomyces cerevisiae* induce changes in the permeability of the membrane; by forming ion channels an uncontrolled leakage of protons, potassium ions, amino acids and ATP molecules will occur causing cell death (Marquina et al. 2002). Alternatively, the *S. cerevisiae* K28 toxin releases a signal that results in inhibition of DNA synthesis, which produces the lethal effect (Liu et al. 2015). Several authors explain the killer activity that *Kluyveromyces lactis* exhibits towards *Saccharomyces cerevisiae* as an attack on the tRNA leading to cell death (Frohloff et al. 2001; Jablonowski and Schaffrath 2007; Jablonowski et al. 2006). Additionally, KP4 toxin produced by *Ustilago maydis* inhibits growth and division of susceptible cells by blocking calcium uptake (Gage et al. 2001).

Debaryomyces hansenii is a haploid yeast that reproduces by multilateral budding (Breuer and Harms 2006) and is an extremophile, one of the most halotolerant yeast species, growing in media containing up to 4M NaCl (Breuer and Harms 2006). It was originally isolated from saline environments (Kumar et al. 2012) and is the most common yeast found in cheese (Banjara et al. 2015) and in dry–cured meat products (Andrade et al. 2009). *D. hansenii* can also be found in other environments with low water activity; hence its association with soil, fruits, wine and beer (Breuer and Harms 2006; Kumar et al. 2012).

Debaryomyces hansenii exhibits the killer phenotype against strains of *Candida albicans* and *Candida tropicalis in vitro* (Banjara et al. 2016) and it has been found to tolerate killer toxins from *Kluyveromyces lactis*, *Candida zeylanoides* and *Trichosporon cutaneum* (Fukuhara 1995; Nout et al. 1997). The genetic basis of the killer activity in *Debaryomyces hansenii* remains unexplored. Gunge reported the presence of three

plasmids, pDHL1, pDHL2 and pDHL3, in *D. hansenii* (Gunge et al. 1993), and discovered that plasmid pDHL3 is fundamental for pDHL1 and pDHL2 replication (Fukuda et al. 1997). Killer yeasts can be cured of plasmid-encoded killer phenotype by a series of methods that include exposure to high temperatures, ultraviolet light and cycloheximide (Fukuhara 1995; Santos et al. 2002). Assays performed on *D. hansenii* strains suggest that the killer character in *D. hansenii* is not cytoplasmically encoded, but chromosomally. Corredor and colleagues reported high genome variability between strains of *D. hansenii* based on chromosomal rearrangements and repeated and deleted sequences (Corredor et al. 2003), and Banjara's work agrees in so far as *D. hansenii* isolates from cheese showed consistent, reproducible variation in killer toxin activity (Banjara et al. 2016).

Santos and Marquina (Santos et al. 2002) described the killer toxin produced by *Debaryomyces hansenii* as a low molecular weight secreted protein, and identified 1→6-β-D-glucan as the cell wall binding site for this killer toxin. It is unknown if additional toxins may exist and how the killing mechanism of these toxins is achieved. Furthermore, Marquina and colleagues (Marquina et al. 2001), and Banjara's work revealed that the killer activity is pH and temperature dependent, with optimum conditions at 20°C and pH 4.5 (Banjara et al. 2016).

It is evident that more research is needed to understand the mechanisms by which *Debaryomyces hansenii* mycocins achieve the lethal effect, so as to take advantage of its full biotechnological potential. In this study we intend to explore the genome so as to be able to look for possible regions or genes that could be responsible for toxin production.

3.3 Materials and Methods

Organisms, growth media and culture conditions

YEPD rich media (1% yeast extract, 2% peptone, 2% dextrose) at room temperature was used to grow 10 selected *Debaryomyces hansenii* strains – seven killer and three non-killer – previously isolated from different type of cheeses (Banjara et al. 2016). *D. hansenii* strains have been deposited at the CBS-KNAW Fungal Biodiversity Centre – an institute of the Royal Netherlands Academy of Arts and Sciences (see **Table 3.1** for *Debaryomyces hansenii* strains and accession numbers).

DNA isolation

High quality DNA extraction for sequencing of *Debaryomyces hansenii* strains, and for amplification of the *Candida albicans* hexokinase gene (*HEX1*) was accomplished using a modified version of the QIAGEN Genomic Tip 100/G and QIAGEN buffer methodology (QIAGEN, Germantown, MD). The yeast protocol from the QIAGEN Genomic DNA Handbook was followed with the additional use of 65–70°C incubation while pelleting, and the addition of beads, sand and sonication in the protease pelleting step to increase yield. DNA extraction for PCR assays was performed using a modified version of the Bust n' Grab method (Harju et al. 2004), in which the freezing (-80°C for 5 minutes) and heating (95°C for 1 minute) steps were repeated twice.

Genome sequencing and analysis

Illumina sequencing, 2 x 250 paired end reads to an estimated 50X coverage, was performed on 7 killer and 3 non-killer, arbitrarily selected, *Debaryomyces hansenii* strains at Michigan State University, Research Technology Support Facility (RTSF). Sequences have been deposited with the National Center for Biotechnology Information (**Table 3.1**). Quality controlled reads were assembled with SPAdes (Bankevich et al. 2012) version 3.1.0 with kmers 21, 33, 55, and 77. Scaffolds longer than 1 kb were retained for downstream analysis. Using core eukaryotic genes, all genomes were estimated to be >94.35% complete (Parra et al. 2007). Genomes were compared in a pairwise fashion using Mugsy (Angiuoli and Salzberg 2011). Outputs from Mugsy were parsed with custom perl scripts to identify genome regions exclusive to killer or non-killer strains. The *Candida* genome database (http://www.candidagenome.org), NCBI (http://www.ncbi.nlm.nih.gov), and Génolevures (Sherman et al. 2009) were used to assign putative functions to sequences of interest.

Table 3.1 *Debaryomyces hansenii* strains, killer toxin activity, accession numbers and source of isolation.

Strain ID	CBS accession number	BioSample Objects	Cheese	Killer Activity
Dhans-68	14260	SAMN04420253	Queso Authentico	–
Dhans-72	14261	SAMN04420254	Hennings Colby	–
Dhans-76	14269	SAMN04420255	Cheddar (Black Creek Smooth and Creamy)	–
Dhans-220	14262	SAMN04420256	Italian Bel Paese (Galbani)	+
Dhans-237	14264	SAMN04420257	Italian Bel Paese	+
Dhans-242	14266	SAMN04420258	Parmesan (Reggiano)	+
Dhans-246	14265	SAMN04420259	Raclette (Grand Cru)	+
Dhans-255			Provolone Cheese (Dilusso's Wisconsin)	–[1]
Dhans-274	14267	SAMN04420260	Blue cheese (Roth Kase Minis)	+
Dhans-276	14268	SAMN04420261	Blue cheese (Roth Kase Minis)	+

+ = Killer positive, presence of an inhibition zone

– = Killer negative, no inhibition zone observed

[1]Dhans-255 was originally identified as killer (Banjara et al. 2016), but was not observed to kill in our assays. It also exhibited morphological variation from the other *D. hansenii* strains and the DNA sequence quality was low, so this strain was excluded from further analysis and was not deposited with CBS or NCBI.

Construction of the gene knockout cassettes

Polymerase chain reaction (PCR) was used to assemble gene knockout cassettes by fusing noncoding DNA upstream and downstream of the gene of interest (genes unique to either killer or non-killer strains) with DNA encoding the *Candida albicans HEX1* gene, following the protocol outlined by Hallen and Trail (2008). The 1000 bp upstream and downstream of genes of interest (**Table 3.2**) were downloaded from Génolevures (Sherman et al. 2009) and trimmed as necessary to ensure these regions contained no predicted proteins. Primers were designed using Lasergene Version 12.0 (DNASTAR, Madison, WI) (**Table 3.4**), and the presence of primer sequences in the relevant *D. hansenii* strains (killer or non-killer, as appropriate) was confirmed using SeqMan NGen version 12.0 (DNASTAR). The 5' ends of primers nearest to the targeted gene (upstream reverse primers and downstream forward primers; **Table 3.4** and **Figure 3.2**) included an additional 17 to 23 nucleotides complementary to the *C. albicans HEX1* gene.

PCR reactions for amplification of noncoding DNA regions (upstream and downstream) were performed with the following conditions: initial denaturation at 95 °C for 3 min, followed by 34 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min, and a final extension step of 72°C for 5 min. Each reaction contained 12.5 μl of Maxima Hot Start Green master mix DNA polymerase (ThermoScientific), 2 μl of each oligonucleotide primer, 7.5 μl of molecular biology-grade water and 1 μl DNA template for a final volume of 25 μl. PCR was performed on Dhans-237 and Dhans-274 to amplify regions unique to killer strains, and on Dhans-68 and Dhans-76 for regions unique to non-killers. *HEX1* was amplified from *C. albicans* SC-1543 using 0.25 μl of TaKaRa Ex

Taq polymerase, 5 µl of 10X Ex Taq Buffer (Mg^{2+} plus), 4 µl of dNTP Mixture, 5 µl each of primers 1 and 2 (**Table 3.4)**, 28.75 µl of molecular biology-grade water, and 2µl DNA template for a final volume of 50 µl), using the amplification conditions described above.

Table 3.2 Chosen genes and their predicted function.

ID of Chosen Regions	Primer ID	Conserved Domains
DEHA2D01056g	D1056	The Major Facilitator Superfamily
DEHA2G14916g	DoubleGene	Multidrug resistance protein
DEHA2G14894g	DoubleGene	ABC-2 type transporter
DEHA2E00220g	E220	No prediction
DEHA2G00110g	G110	Cytochrome P450

ID of chosen regions is the gene ID from Génolevures, and is read as follows: DEHA2D01056g is *Debaryomyces hansenii* (strain CBS 767), sequence build 2, chromosome D, gene 01056, from genomic DNA. Primer ID is derived from the chromosome and gene number, except for "DoubleGene": genes G14916 and G14894 are adjacent, so primers were designed upstream and downstream of the pair, to knock both out simultaneously.

PCR products from the upstream region and the *HEX1* gene were combined with an additional round of PCR using primer 1 and upstream forward primers (primers 5, 9, 13, and 17). Downstream region and *HEX1* gene were combined with an additional round of PCR using primer 2 and downstream reverse primers (8, 12, 16, and 20). Fusion of the upstream-*HEX1* and downstream-*HEX1* was achieved using primers 5 and 8, using an

adaptation of the protocol for Expand Long-Template Polymerase (Roche Applied Science, Indianapolis, IN), as outlined in **Table 3.3**. PCR mixture for amplification of *Candida albicans HEX1* gene and for assembling the knockout cassette contained 0.25 µl of TaKaRa Ex Taq polymerase, 5 µl of 10X Ex Taq Buffer (Mg^{2+} plus), 4 µl of dNTP Mixture, 5 µl of each oligonucleotide primer, 28.75 µl of molecular biology-grade water, and 2µl DNA template for a final volume of 50 µl. Bands were purified as needed using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, WI) following manufacturer's instructions. All amplifications were carried out on a T100 Thermal Cycler (Bio-Rad, Hercules, CA, USA) and PCR products were visualized by ethidium bromide staining on an agarose gel. **Figure 3.2** shows a schematic of this protocol.

Table 3.3 PCR expansion protocol

PCR stage	Temperature	Time
Denaturation	95°C	4 min
Denaturation	95°C	30 sec
Annealing	52°C	30 sec
Extension	72°C	2:30 min
GOTO step 2		10 cycles
Denaturation	95°C	30 sec
Annealing	52°C	30 sec
Extension	72°C	2:30 min +5 s per cycle
GOTO step 6		25 cycles
Extension	72°C	7:00 min
Hold	4°C	∞

3.4 Results and Discussion

High quality DNA from *Debaryomyces hansenii* killer (**Figure 3.1**) and non-killer strains (not shown) was successfully extracted and used for whole genome sequencing.

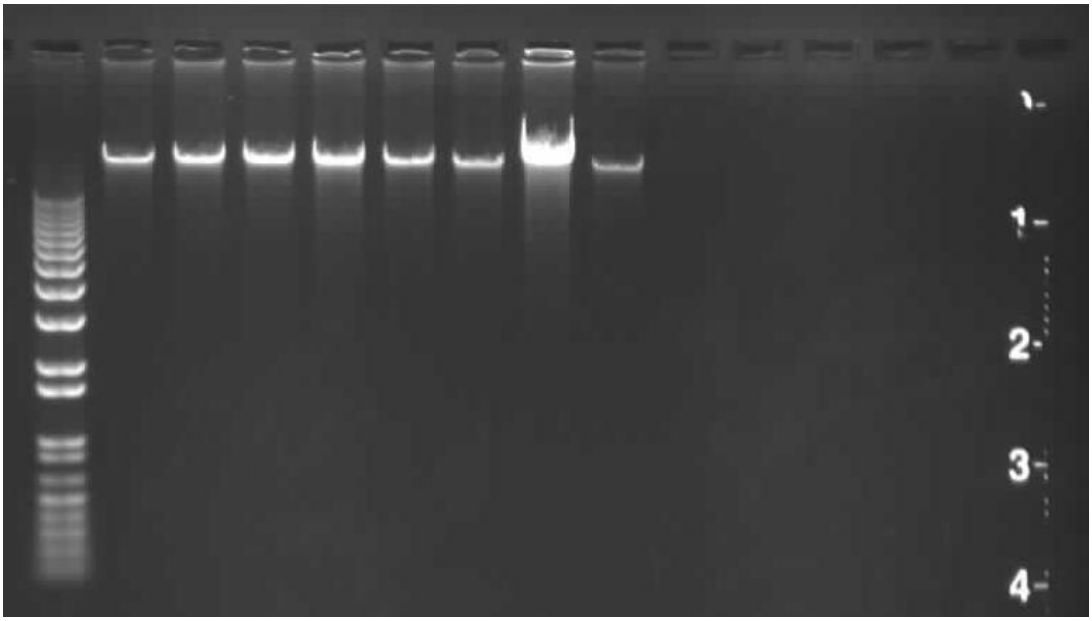

Figure 3.1 DNA isolation. High-quality DNA extraction from *Debaryomyces hansenii* strains. From left to right, killer strains Dhans-242, Dhans-220, Dhans-274, Dhans-276, Dhans-265 (not sequenced), Dhans-246, Dhans-255 and Dhans-237.

Genome analysis identified 48 DNA regions unique to all sequenced killer strains and missing from non-killer strains, and 32 regions present in all non-killers and missing from killer strains, ranging from 31-3434 bp. While single nucleotide polymorphisms (SNPs) and other genetic features may influence killer status, we chose to focus on larger differences involving gene presence and absence. The *Candida* genome database (http://www.candidagenome.org) was used to find the designated regions where the

identified sequence was localized. Subsequently, NCBI (http://www.ncbi.nlm.nih.gov) was used to find the predicted functions or conserved domains of the sequences (**Table 3.2**). We found four distinct regions that look promising, that include a predicted member of the major facilitator superfamily (MFS), which are a group of transporters (Pao et al. 1998); an ABC-2 type transporter (Reizer et al. 1992); cytochrome P450 which is involved in the production and degradation of toxic compounds (Ogu and Maxa 2000), and a multidrug resistance protein that is known to be involved in the export of toxins (Blackmore et al. 2001). Additionally, we selected one gene that had no predicted function and appears to have no homolog in the databases outside of *D. hansenii*. Génolevures provided the genetic elements of the chosen sequences (Sherman et al. 2009). Finally, primers were built for the chosen regions using DNASTAR software (**Table 3.4**) and overlaps were built to complement the *HEX1* gene from *Candida albicans*, which allows them to fuse in a separate PCR reaction.

Table 3.4 PCR primers designed with DNASTAR® for gene knockout.

Primer no.	Primer ID	Sequence
1	Hex1XformF	5’-cccgatccttggtgctc-3’
2	Hex1XformR	5’-ccccttccttaatttgtgtttgat-3’
3	Hex1InternalF	5’-ccaggggatatgatgttgtag-3’
4	Hex1InternalR	5’-ctgtggagctgggagtatttc-3’
5	E220UpF	5’-acgcagggtaaagtatcaatcca-3’
6	E220UpR	5’-**atcaaacacaaattaaggaaggg**_gtgcgcaagccaaaagtgt-3’
7	E220DownF	5’-**gagcaccaaggatcggg**_tctacgaaatgaatgaccta-3’
8	E220DownR	5’-aatatgtgttctaaccttgatg-3’
9	G110UpF	5’-ctattctccattcgcatcacaac-3’
10	G110UpR	5’-**atcaaacacaaattaaggaagggg**_ccagaaaaagggcagcatagt-3’
11	G110DownF	5’-**gagcaccaaggatcggg**_cgcgccgcatcaacaga-3’
12	G110DownR	5’-aggcaacgagtcccgaacataa-3’
13	D1056UpF	5’-aagccagggatgttagg-3’
14	D1056UpR	5’-**atcaaacacaaattaaggaagg**_ggcggctgctgggtttat-3’
15	D1056DownF	5’-**gagcaccaaggatcggg**_atattaactaaagccgcagagact-3’
16	D1056DownR	5’-aaggggatatgaataacagacaac-3’
17	DoublegeneUpF	5’-gaagccggacagaaggtaga-3’
18	DoublegeneUpR	5’-**atcaaacacaaattaaggaagggg**_tatatgtttaatcggagaa-3’
19	DoublegeneDownF	5’-**gagcaccaaggatcggg**_taaaatcggatgcaataagaaatg-3’
20	DoublegeneDownR	5’-aaggcgctcaatccaactc-3’

Bold segments in the UpR and DownF primers are complementary to the *HEX1* gene from *Candida albicans.*

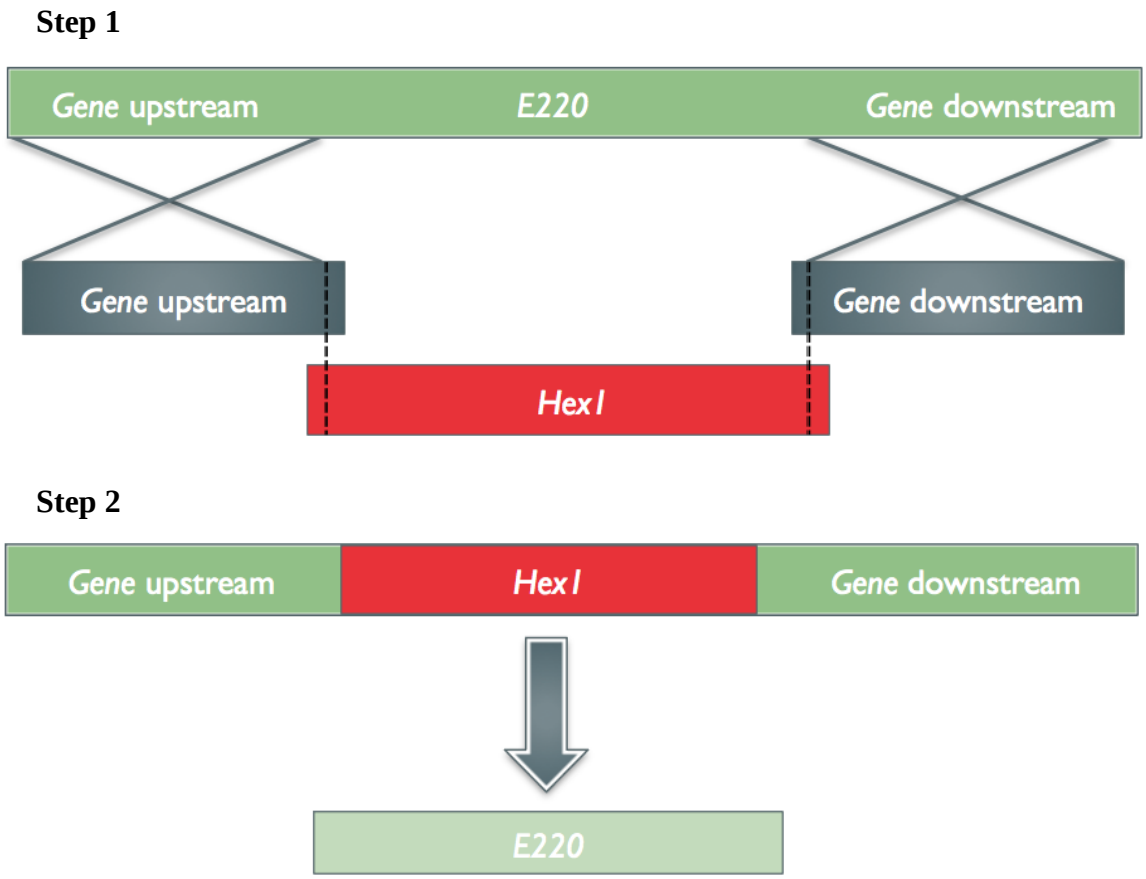

Figure 3.2 Schematic of PCR-based transformation. Amplifying noncoding DNA, both upstream and downstream, of the gene of interest will result in the construction of Δ*E220* mutants. The PCR products of the upstream and downstream regions have overlaps built onto the primers of the *HEX1* gene allowing them to fuse on an additional round of PCR. A separate PCR on *Candida albicans* results in amplification of the *HEX1* gene. An additional PCR is used to fuse together the three product: upstream, downstream and *HEX1*. E220 is replaced by double-crossover integration of the *HEX1* gene into the homologous portion of the genome.

Using *Candida albicans HEX1* gene as a selectable marker will allow us to identify the transformants by their color on CHROMagar™ Candida (Niimi et al. 2001): green colonies for colonies expressing *C. albicans* hexokinase, and purple for wild-type *D. hansenii.* Yeast transformations are usually performed using antibiotic resistance markers (Kaster et al. 1984). However, using hygromycin B as a resistant marker in yeasts is typically difficult. Chand-Goyal and Eckert (1996) found that *D. hansenii* is resistant to the antibiotic and were not able to transform the yeast (Chand-Goyal and Eckert 1996). Nonetheless, Ricaurte (1999) found strains of *D. hansenii* that were susceptible to 100 μg/ml of Hyg and successfully transformed the yeast (Ricaurte and Govind 1999). Effective transformation of *D. hansenii* using *C. albicans HEX1* gene as a marker will be a novelty and will make transformations of non-*albicans Candida* and *D. hansenii* strains uncomplicated due to color identification.

Upstream and downstream regions for the five genes (four genetic regions) we selected have all been amplified from the appropriate killer or non-killer *D. hansenii* strains, and *HEX1* has been amplified from *C. albicans*. To construct the transformation cassette, PCR products from the upstream and downstream regions were amplified together with the *HEX1* PCR product from *C. albicans.* Reactions were performed in which all three products (upstream, downstream and *HEX1*), were present, and in which only two products were present (upstream and *HEX1*, downstream and *HEX1*). We have assembled the transformation cassette for E220 (predicted protein of unknown function), amplifying the upstream and the downstream region from *Debaryomyces hansenii* strain Dhans-68. The PCR product of the *Hex1* gene has a size of 1,686 bp, the upstream product is 507 bp and the downstream product is 423 bp. Using a GeneRuler 1kb Plus

DNA Ladder from Thermo Scientific, we estimated the size of the products. *C. albicans Hex1* gene estimate size is around 2,000 bp, the upstream region is around 500 bp and the downstream region is around 400 bp (**Figure 3.3**). Two separate PCR reactions were done to combine the upstream region with the *Hex1* gene and the *Hex1* gene with the downstream region. An additional PCR fused the last two products so as to incorporate the *Hex1* gene with the upstream and downstream. This final product presented a size of around 3,000 bp, which is congruent with the expected outcome of 2,616 bp (**Figure 3.4**).

3.5 Future Directions

Experiments are underway to incorporate the transformed DNA into a cell. We are following an adaptation of Hallen-Adams et al. 2011, where the first step is to have enzymes destroy the cell walls of the yeast. Subsequently, 30% PEG 8000 and sorbitol solutions will force the DNA into the cell where, in some cells, it will integrate into the nucleus. A high sugar treatment for 24 hours will allow the cell walls to re-form and plating in CHROMagar™ will allow us to identify transformed colonies by their green color (Hallen-Adams et al. 2011).

Preliminary results show that using a high sugar solution results in an excessive amount of yeast recovery, and YEPD media seems to be more adequate for yeast cells regeneration. Additionally, a spread plate technique (100 μl) in CHROMagar™ results in a complete growth of the yeast in the plate, where isolation of a single colony is impossible. A streak plate technique has shown to be more adequate for isolation of colonies. If green colonies are found the next step will be to confirm the presence of the

HEX1 gene performing a PCR reaction with primers 3 and 4, and using up- and downstream primers to confirm the presence of the transformed DNA. Furthermore, southern blot will help us to confirm the presence of specific DNA segments in our samples. If green colonies were not present, electroporation would be our next go to transformation protocol for incorporation of the construct into the cell.

Once the deletion/insertion of genes is confirmed, killer assays will be performed against *Candida albicans* and *Candida tropicalis*. These assays will help us establish the relationship between the gene of interest and the killer toxin activity. If deleting a gene on a killer strain results in the absence of the killer phenotype, this suggest that the gene deleted is somehow involved with the killer toxin production. Studying the genetic basis of these toxins allows us to understand how the killing mechanism works, and could even tell us the gene or genes responsible, if any, for the toxin production.

3.6 Acknowledgments

We would like to acknowledge Nabaraj Banjara for sharing cultures. We also want to express our deepest gratitude to Rohita Sinha and Christopher Anderson for their academic collaboration and work in the assembly and genome analysis of the strains.

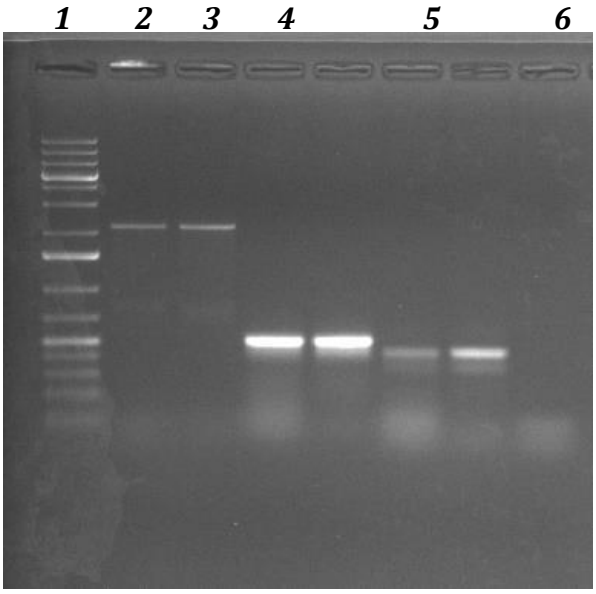

Figure 3.3 PCR products. A 1kb Plus DNA Ladder (**1**) was used to determine the size of the products. *Candida albicans HEX1* gene (**2** and **3**) is close to ~1,686 bp. The upstream (**4**) and downstream (**5**) regions of strain Dhans-68 have a size of ~507 and ~423 bp respectively. Lane **6** shows the water control.

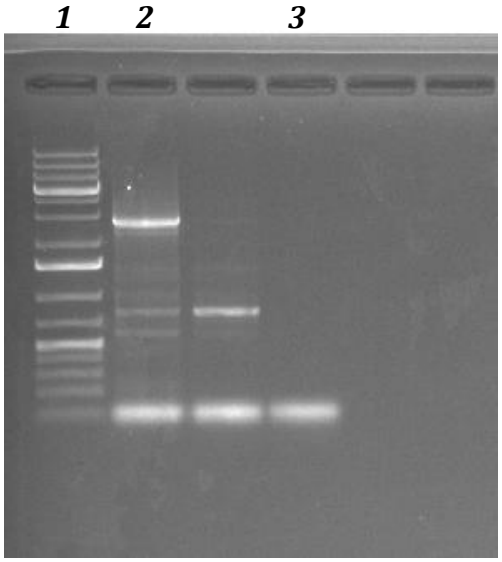

Figure 3.4 Gene replacement cassette. A 1kb marker (**1**) was used to determine the size of the products. Lane **2** shows the product from the Dhans-68 transformation (~3k bp) and lane **3** represents the water control.

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