Glucose induced germ tube formation in *Candida albicans*

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ABSTRACT

Candida albicans; is an opportunistic fungal pathogen that can cause a wide range of superficial and systemic infections. One of the many factors that have been implicated in *C. albicans* success as a pathogen is its ability to reversibly switch between a yeast form and a hyphal form (dimorphism). The dimorphic switch is triggered by a wide variety of stimuli which include temperature alone, pH alone, and serum. Serum is a potent inducer of germ tube formation and remains the medium of choice for rapid identification of *C. albicans* from other non-*albicans Candida* species. Recently it was shown that, in serum, glucose is the primary inducer of germ tubes in *C. albicans* strain A72 (Hudson and Farley, unpublished). In this study the ability of glucose, dialysed serum and serum filtrate to induce germ tube formation in a randomly chosen panel of clinical isolates of *C. albicans* was studied, and the role of two putative glucose receptors and a putative glucose transporter in the transduction of the glucose signal was investigated.

Dialysed serum (molecular weight, > 10 kDa) was less effective ($P > 0.05$, Students *t*-test) at inducing germ tube formation than serum. The addition of exogenous glucose alone to dialysed serum restored its ability to induce germ tube formation levels to those seen in serum in seven of the nine clinical isolates tested. Serum filtrate (molecular weight, < 10 kDa) induced germ tubes to levels indistinguishable from those seen in serum ($P > 0.05$, Students *t*-test) in all but one of the clinical isolates tested. Buffered glucose was also able to induce germ tubes in all the clinical isolates tested and the percentage germ tube formation was not statistically significantly different from that obtained with serum in ten out of sixteen clinical isolates tested. The addition of urea to these assays had no statistically significant effect on the induction of germ tube formation.

It was proposed that the induction of germ tube formation by glucose was mediated by a surface receptor and therefore the *C. albicans* genome was examined for genes encoding putative glucose receptors. Identified as possible receptors were orf19.1944 and orf19.5962. Orf19.3668, a putative glucose transporter, was also examined because its expression had been reported to increase during serum induced germ tube formation. Strains carrying homozygous deletions of each ORF were made and the

phenotypes of the mutants investigated. None of the ORFs were found to be involved in glucose or serum mediated germ tube formation. However, orf19.1944 was shown to play a role in germ tube formation under embedded conditions.

ABBREVIATIONS

BLAST	Basic Local Alignment Search Tool
kDa	kilodaltons
OD	Optical density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
SOSUI	"Japanese" means "Being Hydrophobic" Transmembrane Prediction
TMHMM	Transmembrane Hidden Markov Model
WT	Wild Type

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CHAPTER ONE: INTRODUCTION

1.1 Epidemiology of *Candida albicans*

Candida albicans is an asexual; diploid yeast and a normal part of the indigenous human microbial flora (Marr *et al.*, 2003). An opportunistic pathogen, it can exploit changes in the host microbial environment caused by events such as prolonged courses of antibiotics or a decline in host immunocompetency caused by conditions such as Acute Immune Deficiency Syndrome (AIDS), cancer, and drug treatment following organ transplant, to become pathogenic (Lischewski *et al.*, 1999; Marr *et al.*, 2000). The incidence of these infections has increased rapidly in recent years due to a rise in the number of immunocompromised patients, drug resistant strains and lack of knowledge about its pathogenicity factors (Wroblewska *et al.*, 2002; Bodey *et al.*, 2002). Epidemiology studies have begun to provide a greater understanding of how *C. albicans* has become such a successful pathogen, through the use of molecular tools such as Ca3 fingerprinting and bioinformatics (Pujol *et al.*, 2002; Schmid *et al.*, 1995; Giblin *et al.*, 2001). The majority of infections are caused by one of three clusters of clinical strains, which has evolved a general-purpose genotype that may have arisen from clonal adaption to frequent environmental changes (Schmid *et al.*, 1999). This general-purpose genotype has allowed *C. albicans* to successfully adapt to multiple environments, and out-compete many other microorganisms by providing it with an impressive arsenal of pathogenicity factors. These include adherence to host tissues, secreted hydrolases, and dimorphism (the ability to reversibly switch between a yeast form and a hyphal form) (Fu *et al.*, 1999; Phan *et al.*, 2000; Naglik *et al.*, 2003).

1.2 Pathogenicity factors

1.2.1 Adherence to host tissues

The first stage of infection by *C. albicans* is adherence to host tissues through numerous cell surface adhesins, without this ability *C. albicans* would not be able to colonise the host (Fu *et al.*, 1999). Studies to isolate putative adhesins in *C. albicans* have been facilitated through work done on non-adhesive avirulent spontaneous mutants, and the identification of proteins that bind to several extracellular matrix

(ECM) proteins of mammalian cells, such as fibronectin (FN), laminin, fibrinogen and collagen type I and IV (Gaur and Klotz, 1997; Hoyer, 2001). The ability to bind ECM proteins like FN, laminin and collagen I and IV lead to the discovery of the adhesin gene *INT1*, which encodes the first discovered non-mammalian integrin (Gale *et al.*, 2001). Homozygous deletion mutants of *INT1* were shown to be less adherent to human epithelial cells, defective in germ tube formation and less virulent (Gale *et al.*, 2001). Another protein that contributes to cell adhesion is Hwp1p. Hwp1p is a germ tube-hyphae specific, outer surface mannoprotein that serves as a substrate for transglutaminases (TGases', host enzymes that catalyse the covalent crosslinking of tissues proteins via the amino acid residues glutamate and lysine). It has long been known that the hyphal form of *C. albicans* binds host tissue surfaces stronger than the yeast form. TGases' can catalyse the crosslinking of recombinant Hwp1p to ^{14}C-putrescine (Tsuchimori *et al.*, 2000; Staab *et al.*, 1999). The importance of this protein was demonstrated with a homozygous deletion mutant of *HWP1* which caused reduced mortality in mice, germinated less readily in the kidneys of infected mice and caused less endothelial cell damage (Tsuchimori *et al.*, 2000). Finally, a family of proteins that aids cell adhesion is the Agglutinin-Like Sequence (*ALS*) family, which is composed of at least nine proteins that structurally resemble the *Saccharomyces cerevisiae* cell-surface adhesion, alpha-agglutinin. The *ALS* family is a member of the immunoglobulin superfamily (Hoyer, 2001). The *ALS* genes are differentially regulated between the yeast and hyphal stages, and it has been postulated that each is expressed at a certain stage of growth to confer a range of adhesive advantages in pathogenesis (Hoyer and Hecht, 2000). To date, functional analysis of the *ALS* family has only been carried out on Als1p and its allelic counterpart Ala1p (Gaur and Klotz, 1997; Fu *et al.*, 1998). Heterologous expression of these genes in the normally non-adherent yeast *S. cerevisiae* confers an adherence phenotype upon it, to host cell surfaces and ECM proteins (Gaur and Klotz, 1997). Studies of the *ALS* family have also revealed that it has many significant parallels to the Secreted Aspartic Proteinase (*SAP*) family of *C. albicans*. Each is regulated by similar mechanisms, there are a similar number of genes, and both gene families are largely co-localised on the same chromosomes (Hoyer, 2001).

1.2.2 Secreted Hydrolases

The *SAP* family is the largest, containing 10 members thus far, and the most extensively studied secreted hydrolase group in *C. albicans*. Their proposed roles in pathogenesis include the digestion of host proteins for nutrient supply, the evasion of host immune defences by degrading immunoglobulin and complement proteins, and degradation of structural barriers that the host uses to prevent invasive infection (Schaller *et al.*, 1999; Staib *et al.*, 2000). Experimental study of the *SAP* gene family has uncovered an ever increasing complexity to their regulation, which seems to suggest that the various members have distinct roles in host colonisation and invasion (Staib *et al.*, 2002; Staib *et al.*, 2002; Ibrahim., 1998). Research so far shows that *SAP1* and *SAP3* are regulated during phenotypic switching between the white and opaque forms of strain WO-1 (White *et al.*, 1993). *SAP2* is expressed in the yeast form, and the *SAP4, SAP5,* and *SAP6* genes are observed only at neutral pH during the serum induced dimorphic switch from the yeast to hyphal form (White and Agabian, 1995; Chen *et al.*, 2002). The Sap4p, Sap5p and Sap6p isoenzymes also play an important role in maintaining systemic infection by the induction of *SAP2*. *SAP8* expression is temperature regulated, and *SAP9* and *SAP10* are constitutively expressed in both the yeast and hyphal forms (Monod *et al.*, 1998). A mutant lacking all ten *SAP* genes has not been isolated thus far but the role in pathogenesis of several genes has been deduced using *SAP*-deficient mutants. *SAP1-3* are involved in the infection of oral and vaginal mucosal membranes and individual and concurrent knockout mutants are less virulent (Schaller *et al.*, 2000; Hube *et al.*, 1997). Strains lacking *SAP4-6* caused less tissue damage and invasion in peritoneal infections, and were also less virulent (Sanglard *et al.*, 1997). Inhibition of the Sap proteinases using the aspartic proteinase inhibitor pepstatin A prevented initial penetration of *C. albicans*, and damage to tissues in oral, vaginal and skin experimental infection models and adherence to epithelial cells (Schaller *et al.*, 2000; de Bernardis *et al.*, 1999). Other secreted hydrolytic enzymes which have been implicated in the pathogenesis of *C. albicans* include the phospholipases. The phospholipase *PLB1* has been studied extensively, and research shows that homozygous deletion mutants have attenuated virulence in murine models (Leidich *et al.*, 1998) and reintroduction of *PLB1* back into *C. albicans* restores wild-type virulence (Mukherjee *et al.*, 2001).

1.2.3 Morphological Variation

Certain strains of *C. albicans* are able to undergo a reversible morphological change known as phenotypic switching. An ability that aids in evasion of the host immune defences, increases drug resistance, and allows survival in a range of different environments (Lan *et al.*, 2002; Kvaal *et al.*, 1999; Vargas *et al.*, 2000). The two best studied strains are 3153a and WO-1. Strain 3153a alternates between at least seven different colony morphologies, and can also switch between colonies with and without dense myceliation (Vargas *et al.*, 1994). Other phenotypic consequences of this switch were reduced adherence to buccal epithelium and changes in hyphal formation rates (Vargas *et al.*, 1994). Strain WO-1 alternates between white hemispherical colonies, designated white (W), and grey flat colonies, designated opaque (O). W/O phenotypic switching also affects the size and shape of cells, their ability to form hyphae, adhesion and drug susceptibility (Balan *et al.*, 1997; Zhao *et al.*, 2002). There is also a distinct metabolic difference between W and O cells that has been linked to a phenotype selection bias at anatomical sites with different nutrient sources (Lan *et al.*, 2002). The molecular basis of phenotypic switching in *C. albicans* is not well understood, however recent work has shown a primary role for *SIR2* and *EFG1* in its regulation (Sonneborn *et al.*, 1999; Perez-Martin *et al.*, 1999).

One of the more extensively researched morphological changes in *C. albicans* and a distinguishing feature identifying it from other non-*albicans* species of *Candida*, is its ability to undergo a reversible transition from a yeast form to a hyphal form (Leberer *et al.*, 1997). Both forms are found at sites of infection, but it is widely considered that the hyphal form, which in its early stage of formation is termed a germ tube, confers certain advantages that the yeast form does not, such as tissue penetration, thigmotropism (surface morphology sensing) and host immune evasion (Brown *et al.*, 1999). Strains that are unable to form hyphae have attenuated virulence (Liu *et al.*, 1994; Rocha *et al.*, 2001). The morphological transition from the yeast form to the hyphal form is triggered by a wide variety of stimuli which include, temperature, pH, various complex media such as Lee's and Soll's, and serum (Nantel *et al.*, 2002; Lee *et al.*, 1975; Ramon *et al*, 1999).

1.3 Molecular biology of germ tube formation

1.3.1 Cell cycle regulation

The *C. albicans* cell cycle is shown in Figure 1. In normal cell division the daughter cell appears during the G1 phase of the cell cycle and continues to grow through S phase, where DNA replication occurs, G2 phase and into M phase, where the segregation of the DNA into a genetically identical mother and daughter cells occurs (Hazan *et al.*, 2002b). The G1 and G2 phases during the cell cycle act as delay mechanisms to allow the cells to grow and double their complement of proteins and organelles, and allow monitoring, to check for favourable, external environmental conditions before cell division can proceed. These cell cycle checkpoints, which lie at the G1 – S phase and G2 – M phase junctions, are regulated by the Cdc28p protein kinase, which associates with different sets of cyclins during the cell cycle allowing it to coordinate the execution of the cell cycle (Segal *et al.*, 1998). In *S. cerevisiae* phosphorylation of Cdc28p by Swe1p halts the normal cell cycle and increases cell elongation and filamentation, indicating that morphogenesis involves cell cycle regulation by Cdc28p (Eddington *et al.*, 1999). In *C. albicans*, yeast and hyphal cells display similar dynamics of phosphorylation and dephosphorylation of Cdc28p. In addition, cell cycle rearrangement of the actin cytoskeleton is similar between the two morphological states, suggesting that when *C. albicans* undergoes the dimorphic switch and germ tube formation occurs normal cell cycle progression is not altered (Hazan *et al.*, 2002b). However, as the germ tube elongates, and the septa are laid down, the progression of the normal cell cycle in the subsequent, sub-apical cells alters (Hazan *et al.*, 2002b). Sub-apical cells do not immediately re-enter the cell cycle instead they become arrested in G1 phase, whilst the apical cell continues through the normal cell cycle (Hazan *et al.*, 2002b). The sub-apical cells can re-enter the cell cycle, but only once they have increased their cytoplasmic mass to the point where they have enough nutrients to continue (Barelle *et al.*, 2003).

1.3.2 Site selection

Normal yeast cell division in *C. albicans* occurs by budding. When the daughter cell reaches maturity it breaks off from the mother cell and the process continues.

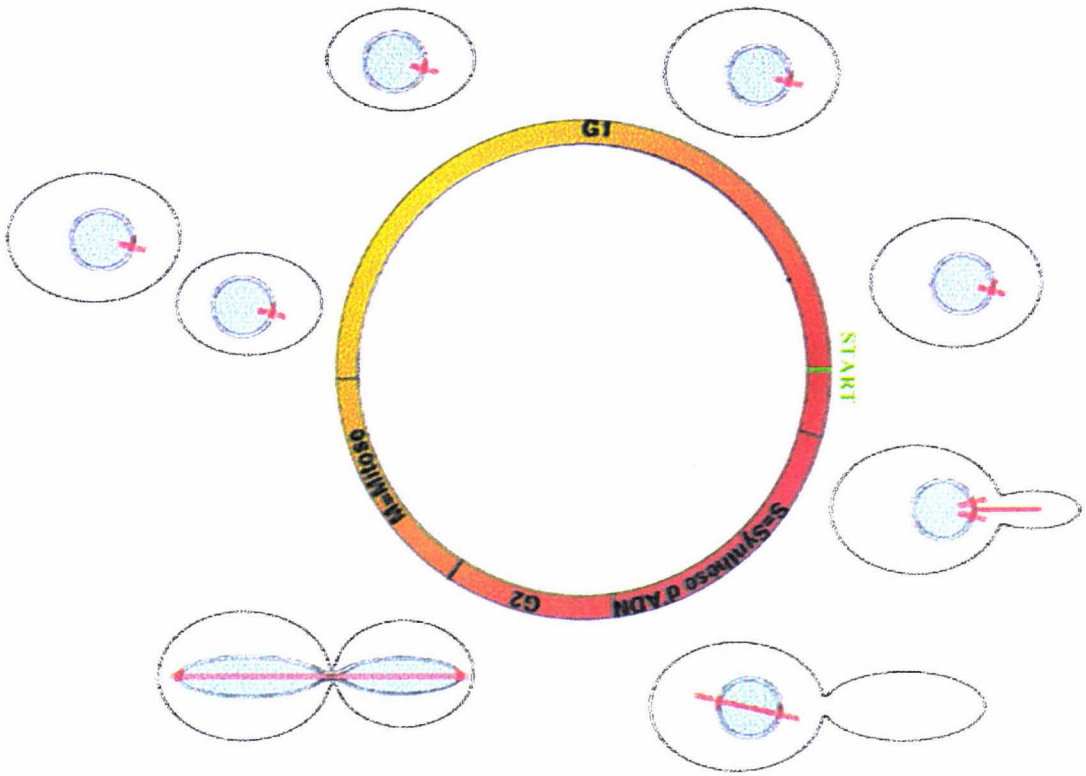


Figure 1 The Cell Cycle of *Candida albicans* (adapted from **http://www-dsv.cea.fr/thema/sbgm/web_sbgm/pages_sbgm_fr/sbgm_groupes_fr/cycle_cellulaire_fr.htm)**

Whilst growing in yeast form *C. albicans* develops buds in an axial pattern. However in the switch to germ tube formation, site selection changes to a lateral or polar pattern (Herrero *et al.*, 1999). Site selection in *S. cerevisiae* begins with the formation of a ringed cytoskeletal structure made up of four septins, Cdc3p, Cdc10p, Cdc11p and Cdc12p, called the 10 nm filament, at the site of the nascent bud or pseudohypae (Kim *et al.*, 1991). In *S. cerevisiae* targeting of these septins is regulated, in part, by four protein kinases, Elm1p, Hsl1p, Gin4p and Kcc4p (Barral *et al.*, 1999). In *C. albicans* homologs to to all four septins have been identified, but not of the four protein kinases. The septins have been shown to localise and interact in a

manner similar to that found in *S. cerevisiae*, during budding and pseudohyphal growth (Warenda and Konopka, 2002). However, the pattern of septin localisation in the emerging germ tube is different. At the site of germ tube emergence, the septins appear to be diffuse, and not form the distinct ring observed in the other morphologies (Warenda and Konopka, 2002). Only when the germ tube has extended at least 10 – 15 μm and a new septum been laid down does the typical septin ring structure reappear. This then continues as new septa are laid down throughout the growth of the hyphae. The diffuse nature of the septins during germ tube formation appears to be due to changes in septin regulation during growth. The role of septins in germ tube formation and hyphal growth is still to be determined, but they may play a role in promoting proper hyphal morphogenesis by recruiting proteins such as Int1p, which has been shown to bind septins and is necessary for hyphal morphogenesis under some conditions (Gale *et al.*, 2001). Septins may also function to define the shape of the hyphae by acting as a boundary domain that helps to restrict actin to the growing tip. Once the 10 nm filament in *C. albicans* has formed, transmission of the positional information may occur through a protein scaffold homologous to the Bud2p, Bud5p and Rsr1p complex found in *S. cerevisiae* (Park *et al.*, 1997). Once germ tube site selection has occurred physiological changes begin to take place within the mother cell and at the nascent germ tube site, allowing morphogenesis to occur.

1.3.3 Physiological regulation

Once site selection occurs and the nascent germ tube begins to form, distinct physiological changes begin to take place within the mother cell. Cytoplasmic microtubules align themselves to the position of the 10 nm filament and move into the germ tube. The nucleus migrates up into the germ tube, 10 – 15 nm on the cytoplasmic microtubules, and it is here that nuclear division takes place. Then the daughter nucleus migrates forward with the germ tube apex while the parent nucleus returns to the mother cell. It is the plane of nuclear division that determines the site of the first septum. During nuclear migration into the germ tube a large vacuole begins to form rapidly within the mother cell, until the mother cell becomes highly vacuolated (Palmer *et al.*, 2003; Barelle *et al.*, 2003). Vacuolation is most extreme in serum induced germ tubes, but occurs to a lesser extent for germ tubes induced in media that have high concentrations of amino acids or *N*-acetyl-glucosamine. Once the first septum is laid down the mother cell becomes arrested in G1 phase and very little new

cytoplasm is synthesised. Any increase in mother cell volume is accounted for by expansion in vacuolar volume (Palmer *et al.*, 2003). As the germ tube apex continues to expand further, it does so in a linear fashion. This is in contrast to hyphal growth of filamentous fungi, which is exponential. Exponential growth occurs because the expansion of the hypha is restricted to the apex but is fed by an increasing volume of cytoplasm that supplies biosynthetic potential for growth. Whilst in *C. albicans* cytoplasmic volume is constant, thus so are the biosynthetic resources and linear growth occurs (Barelle *et al.*, 2003). As the apex continues to expand nuclear division occurs at regular hourly intervals, and in response, septa are laid down. The sub-apical cells then arrest in G1 phase, like the mother cell, and become highly vacuolated (Barelle *et al.*, 2002). The period of cell cycle arrest can be decreased by the provision of assimilatable forms of nitrogen (Barelle *et al.*, 2002). The polarised growth of the germ tube is maintained by localisation of the actin cytoskeleton at the tip of the hyphae, and subsequent localisation of Cdc42p at this site. Cdc42p is differentially regulated by the cell cycle program and the hyphal morphogenesis program to determine the polarity of the actin cytoskeleton. It is also important for normal cell growth and hyphal formation (Hazan and Haoping, 2002a; Ushinsky *et al.*, 2002). This process is analogous to mating projection formation in *S. cerevisiae*. In addition, neither schmoos nor germ tubes have constrictions at their base, both occur in G1 arrested cells, or at least before the G1/S transition, and both have a faint, diffuse septin ring at their base (Warenda and Konopka, 2002). Also both use transmembrane receptors to respond to inducers of germ tube formation. *C. albicans* utilises an amino acid sensor, Csy1p, to sense its environment, co-ordinate amino acid metabolism, and regulate germ tube formation on solid serum and Lee's media (Brega *et al.*, 2004).

1.4 Biochemistry of germ tube formation

The induction signal that stimulates germ tube formation in *C. albicans* is transduced by multiple biochemical pathways that converge to regulate a common set of hyphal specific genes (Lane *et al.*, 2001a). Positive regulatory pathways identified thus far are the mitogen-activated protein kinase (MAPK) pathway, whose response is mediated by the transcription factor Cph1p, and three pathways whose signals all

converge to differentially regulate the transcription factors Efg1p; the cAMP dependent protein kinase A pathway, the Rim101p pH-responsive pathway, and the Cph2p pathway. Efg1p in turn regulates the transcription factors Tec1p and Czf1p. Negative regulatory pathways identified so far are the Tup1p-mediated pathway and the Rbf1p-mediated pathway (Figure 2).

1.4.1 Activators of Germ Tube Formation

1.4.1.1 The Cph1p-mediated mitogen activated protein kinase (MAPK) pathway

In *S. cerevisiae* there are two MAPK pathways that regulate cell morphogenesis, the pheromone response pathway and the filamentation/invasion pathway (Gustin *et al.*, 1998; Csank *et al.*, 1998). The pheromone response pathway governs morphological changes in mating, whilst the filamentation/invasion pathway governs pseudohyphal formation and invasive growth (Gustin *et al.*, 1998).

Both pathways have similar components, and converge to regulate the transcription factor Ste12p (Gustin *et al.*, 1998). In *C. albicans* two related MAPK pathways have been incompletely identified, and analogous to the regulatory pattern in *S. cerevisiae*, both converge to regulate a common transcription factor, the *C. albicans* Ste12p ortholog, Cph1p (Chen *et al.*, 2002). The role of Cph1p was identified when heterologous expression of *CPH1* in strains of *S. cerevisiae* carrying a homozygous deletion of *STE12*, was able to complement both the mating defect of *ste12* haploids and the filamentous growth defect of *ste12/ste12* diploids (Liu *et al.*, 1994). *C. albicans* strains carrying a homozygous deletion of *CPH1* are unable to undergo germ tube formation on solid Spider and SLAD media, but are still able to form germ tubes in liquid media (Liu *et al.*, 1994).

Directly upstream of Cph1p is the divergence between the two pathways, the regulatory kinases Cek1p and Cek2p. Cek1p is orthologous to Kss1p from *S. cerevisiae*, which functions in both the filamentation/invasion and pheromone response pathways in *S. cerevisiae* (Csank *et al.*, 1998). Cek2p is orthologous to Fus3p from *S. cerevisiae*, which is part of the pheromone response pathway regulated by alpha and 'a' mating factors, in *S. cerevisiae* (Chen *et al.*, 2002).

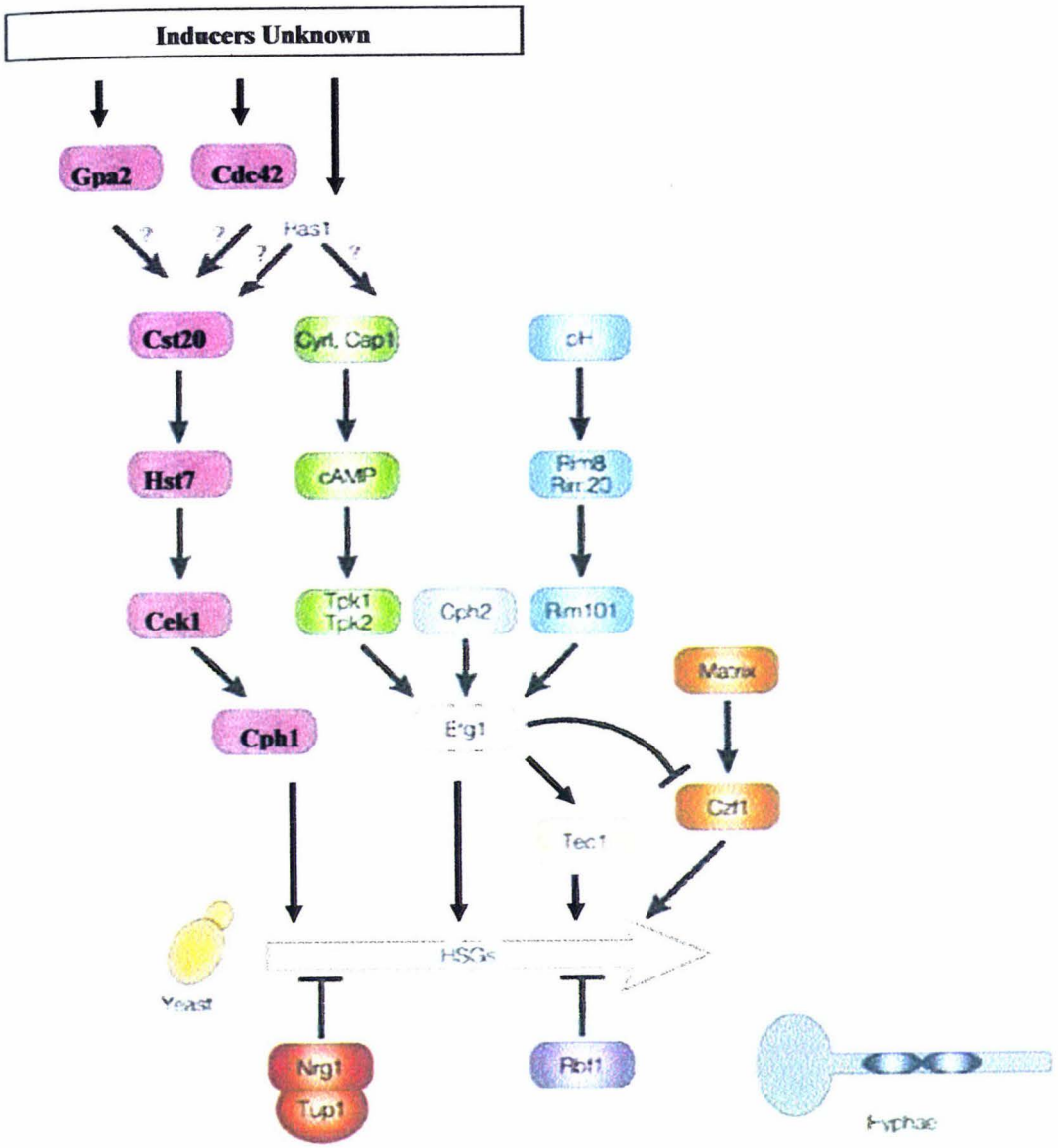


Figure 2 Biochemical pathways regulating germ tube formation in *Candida albicans* (Adapted from Berman and Sudbery, 2002)

Investigation of sexual recombination in *C. albicans*, using artificially generated strains, showed that *C. albicans* has the ability to undergo mating (Chen *et al.*, 2002). This ability was only partially blocked when homozygous deletions of either *CEK1* or *CEK2* were introduced. However when a homozygous deletion of both *CEK1* and *CEK2* was introduced into

MTLa and *MTL alpha* strains mating was completely abolished. Reintroduction of *CEK1* was able to rescue mating (Chen *et al.*, 2002). Therefore, both *CEK1* and *CEK2* function in the same MAPK pathway and regulate mating in *C. albicans*. Strains carrying a homozygous deletion of *CEK1* were unable to undergo germ tube formation on solid Spider and SLAD media, but still formed germ tubes in liquid media. Strains carrying a homozygous deletion of *CEK2* were able to form germ tubes under all hyphal induction conditions tested (Csank *et al.*, 1998; Chen *et al.*, 2002). Thus, in addition to its possible role in mating *CEK1*, functions in the same hyphal induction cascade as *CPH1*, whilst *CEK2* seems to have a redundant role not linked to germ tube formation.

Upstream of Cek1p are three more regulatory kinases, Hst7p, Hst11p and Cst20p, orthologs to Ste7p, Ste11p and Ste20p from *S. cerevisiae* (Kohler and Fink, 1996). *HST7* and *CST20* are the only two to be functionally characterised, with *HST11* identified from the *Candida albicans* genome sequencing project. *HST7* and *CST20* were identified by their ability to restore mating and pseudohyphal growth in strains of *S. cerevisiae* carrying homozygous deletions of either *STE7* or *STE20* (Kohler and Fink, 1996). Strains of *C. albicans* carrying homozygous deletions of one or both genes were unable to form germ tubes on solid Spider and SLAD media, but were still able to form germ tubes in liquid media (Kohler and Fink, 1996).

Upstream of *CST20* are three G-proteins Cdc42p, Ras1p and Gpa2p. Strains of *C. albicans* with a homozygous deletion of *CDC42* are not viable and cease proliferation producing large, round, unbudded, and multinucleated cells (Ushinsky *et al.*, 2002). Placement of *CDC42* under an inducible promoter did, however, allow functional study of its role in serum induced germ tube formation. Cdc42p depletion prior to serum induction trapped cells in the yeast morphology, whilst depletion at the same time as serum induction permitted the initiation of germ tubes that failed to continue extending (Ushinsky *et al.*, 2002). Expression of a hyperactive form of Cdc42p, $Cdc42p^{G12V}$, showed a similar phenotype to strains of *C. albicans* carrying a homozygous deletion of *CDC42*. Deletion of the MAPK regulatory kinase *CST20* suppressed this phenotype and allowed cells to develop normally, suggesting that *CDC42* is a component of the MAPK pathway. Germ tube induction was not tested in this double mutant (Ushinsky *et al.*, 2002). That *CDC42* plays a role in germ tube

formation under serum induction conditions suggests that it may also act in at least one additional pathway, since carrying deletions of any of the other known components of the *C. albicans* MAPK pathway are only defective on solid Spider and SLAD media (Ushinsky *et al.*, 2002; Kohler and Fink, 1996; Chen *et al.*, 2002; Liu *et al.*, 1994).

This is also true for the G-protein Ras1p, which lies upstream or in parallel to Cdc42p in the MAPK pathway. In *S. cerevisiae* there are two functionally redundant *RAS* genes, *RAS1* and *RAS2*, and both can signal through the cAMP pathway, (via the adenyl cyclase encoded by *CYR1*) and the MAPK pathway (through the Raf protein kinase) (Shima *et al.*, 2000). The *RAS1* gene of *C. albicans* was identified by its ability to suppress a viability defect in a strain of *S. cerevisiae* carrying a homozygous deletion of both *RAS1* and *RAS2* (Feng *et al.*, 1999). A strain of *C. albicans* with a homozygous deletion of *RAS1* was unable to form germ tubes under serum induction conditions, and expression of a dominant active *RAS1* caused enhanced germ tube growth (Feng *et al.*, 1999). The addition of cAMP into the media, or over expression of *HST7* and *CPH1* was able to suppress the germ tube induction defect of strains carrying a homozygous deletion of *RAS1* in serum, spider and SLAD conditions (Leberer *et al.*, 2001). Thus Ras1p is a component of the MAPK pathway and the cAMP pathway.

C. albicans GPA2 was identified by its homology to *GPA2* from *S. cerevisiae* in a search of assembly 6 of the *C. albicans* genome. *GPA2* from *S. cerevisiae* is part of the cAMP pathway and regulates cAMP synthesis in response to glucose (Sanchez-Martinez and Perez-Martin, 2002). A strain of *C. albicans* carrying a homozygous deletion of *GPA2* was still able to undergo germ tube formation in serum induction conditions, but not on solid SLAD, Spider and embedded media. This is a phenotype similar to that observed in strains carrying homozygous deletions of components in the MAPK pathway (Sanchez-Martinez and Perez-Martin, 2002). Furthermore, in the *GPA2* the loss of germ tube formation could not be reversed by the addition of exogenous cAMP, but rather by overexpression of the MAPK pathway component *HST7* (Sanchez-Martinez and Perez-Martin, 2002). Thus, in *C. albicans* Gpa2p functions in the MAPK pathway rather than the cAMP pathway. In *S. cerevisiae* the signal transduced by Gpa2p is initiated by the G-protein coupled receptor, Gpr1p (Xue *et al.*, 1998). Gpr1p is proposed to be a dual sensor of glucose and nitrogen

(Kraakman *et al.*, 1999). A similar receptor may interact with Gpa2p, in *C. albicans*, but although orthologs to the pheromone G-protein coupled receptors Ste2p and Ste3p from *S. cerevisiae*, that act in the MAPK pathway, are under investigation, to date none have been functionally characterised.

1.4.1.2 Tec1-mediated pathways

The cAMP/PKA, Cph2p and pH-response pathways all converge to differentially regulate the transcription factor **E**nhanced **F**ilamentous **G**rowth **1** (Efg1p). They do this through posttranslational modification of specific residues in a highly conserved APSES domain, which is a crucial component in morphogenetic regulators from other fungi, such as Sok2p and Phd1p in *S. cerevisiae*. Efg1p is also a member of the basic helix loop helix (bHLH) family of transcription factors, and as such, is a DNA-sequence specific binding protein that binds to an E-box element (5'-CANNTG-3') in the promoter region of the transcription factor Tec1p (Leng *et al.*, 2001; Stoldt *et al.*, 1997). Tec1p is a member of the TEA/ATTS family of transcription factors and it is suggested that once expressed, it is able, together with Efg1p and/or the regulators of Efg1p, to synergistically activate hyphal specific genes, producing a pathway specific response.

1.4.1.3 The cAMP protein kinase A (cAMP/PKA) pathway

Regulation of Efg1p by the cAMP/PKA pathway is predicted to be mediated by two isoforms of a protein kinase A (PKA) catalytic subunit, Tpk1p and Tpk2p, through phosphorylation of a threonine residue found within the APSES domain (Ernst and Bockmuhl, 2001). Both Tpk1p and Tpk2p have distinct and redundant roles in germ tube formation, and seem to be under the control of different cAMP-dependent pathways that determine the cellular activity of the catalytic subunits depending on whether the inducing medium is liquid or solid (Bockmuhl *et al.*, 2001). In liquid induction medium Tpk2p alone activates Efg1p, whilst on solid induction media Tpk1p is the main inducer of Efg1p-mediated germ tube formation. Tpk2p produces a weak Efg1p induction signal on solid media, and also a secondary signal for invasive growth via Efg1p (Bockmuhl *et al.*, 2001, Sonneborn *et al.*, 2000). Efg1p then mediates the solid media, invasive response, through the transcription factor **C**andida **Z**inc **F**inger **1** (*CZF1*) (Brown *et al.*, 1999). The catalytic domain of both Tpk1p and Tpk2p is contained in the C-terminal, whereas the N-terminal of Tpk2p mediates agar

invasion. All PKAs are structurally conserved, consisting of two catalytic subunits that are inactivated by the binding of a homodimer of regulatory subunits. Activation of the catalytic subunits occurs when cAMP, produced by the only adenyl cyclase in *C. albicans*, Cdc35p, binds to the regulatory subunits and dissociates the complex (Rocha *et al.*, 2001). So far no regulatory subunits have been identified or functionally characterised in *C. albicans*. *CDC35* is itself not essential for the survival of *C. albicans*, but it is essential for germ tube formation in conditions that signal through the Cph1p-mediated MAPK pathway and the cAMP pathway (Rocha *et al.*, 2001). Overexpression of the MAPK genes *HST7* or *CPH1*, or the cAMP pathway transcription factor *EFG1* could not restore germ tube formation, suggesting that germ tube induction through the MAPK pathway can only happen during simultaneous elevation of cAMP levels and ligand activation, and that Efg1p is a direct target of the cAMP pathway, through Tpk1p and Tpk2p, which require cAMP activation. Structural analysis of Cdc35p shows that in addition to the ATP-binding catalytic domain, it contains a central Ras1p binding domain, composed of amiphipathic leucine-rich repeats of 23 amino acids (Rocha *et al.*, 2001). Ras1p is predicted to stimulate cAMP levels through its interaction with Cdc35p, and thus being able to interact with both the MAPK pathway and the cAMP pathway (Leberer *et al.*, 2001). Cdc35p is also regulated by the Adenylate **C**yclase **A**ssociated **P**rotein (*CAP1*). Adenylate cyclase associated proteins of other fungi interact with Ras proteins and adenylate cyclase to regulate cAMP levels under specific environmental conditions. Just prior to germ tube emergence in *C. albicans* there is an increase in cytoplasmic cAMP levels. Deletion of *CAP1* diminishes this rise and results in a decreased and delayed germ tube formation in media that activates both the cAMP and MAPK pathways. The addition of cAMP restored germ tube formation (Bahn and Sundstrom, 2001). The fact that *CAP1* mutants could still undergo germ tube formation, albeit at lower and slower levels means that there are other proteins present that can stimulate Cdc35p. This could be Ras1p or some as yet, unidentified Cap protein. Ras1p is the most upstream element identified and functionally characterised so far in the cAMP pathway. To be activated Ras1p is post-transcriptionally modified by the addition of a farnesol group at the C-terminus by a prenyltransferase. This allows Ras1 to become membrane bound and transduce the morphogenetic signal initiating germ tube formation (J. C. D. Green, unpublished). The protein responsible for the prenylation and possible upstream regulation of Ras1p are yet to be elucidated.

1.4.1.4 The Rim101p pH response pathway

One environmental signal that is crucial in regulating germ tube formation *in vitro* and *in vivo* is pH. At acidic pH *C. albicans* grows exclusively in the non-pathogenic yeast form, whilst at alkaline pH, it is most able to form germ tubes and become pathogenic (Barkani *et al*, 2000). A conserved alkaline response pathway has been identified in the fungi *Aspergillus nidulans*, *Yarrowia lipolytica*, and *S. cerevisiae*, and using the components of these pathways as models, the Rim101p pH response pathway was identified in *C. albicans* (Davis *et al.*, 2000a). No putative receptors have been identified so far, but three regulatory components have, Rim8p, Rim20p and Rim101p. When the alkaline pH signal is transduced, Rim8p and/or Rim20p are predicted to associate with a proteolytic complex that activates Rim101p by cleaving the C-terminal, at a conserved PEST sequence (Xu and Mitchell, 2001). The PEST sequence contains a segment rich in proline, aspartate, glutamate, serine and threonine residues that confers susceptibility to ubiquitination and proteasomal degradation, and is also found in the Rim101p orthologs from *S. cerevisiae* and *A. nidulans* (Lamb *et al.*, 2000; Mingot *et al.*, 1999). A key difference is that cleavage in *A. nidulans* occurs at a site far from the PEST region, whilst in *S. cerevisiae* and *C. albicans* cleavage occurs adjacent to the PEST region.(Xu and Mitchell, 2001). Rim101p is a zinc finger transcription factor, and once cleaved into its shortened-active form it regulates the transcription of Efg1p, which in turn regulates Tec1p transcription. Two models exist as to how the rest of the signal is transduced. Tec1p and Rim101p may by themselves, synergistically regulate alkaline pH germ tube formation, or Efg1p may directly regulate germ tube specific genes directly and in cooperation with Tec1p/Rim101p (Lane *et al.*, 2001a). This pathway, however, may not be the only pH response pathway regulating germ tube formation in *C. albicans*. Uncoupling of Rim101p proteolytic activation does not completely bypass the control of germ tube formation by external pH, and *PHR2*, a gene normally repressed by the Rim101p pathway becomes an alkaline inducible gene (Davis *et al.*, 2000b). This unidentified pathway is proposed to act in a positive role to promote germ tube formation, in conjunction with the Rim101p pathway, although other explanations may also be possible.

1.4.1.5 The Cph2 Pathway

Cph2p is a bHLH transcription factor of the Myc subfamily that regulates germ tube formation in a medium specific manner (Lane *et al.*, 2001b). Sequence analysis of all known Cph2p regulated genes shows that many do not contain a Cph2p binding site, but all do contain Tec1p binding sites (Lane *et al.*, 2001b). In addition, deletion of *EFG1* results in a loss of *TEC1* transcription, whilst a deletion of *CPH2* has no effect on *TEC1* transcription (Lane *et al.*, 2001b). It is proposed that Cph2p binds to the promoter region of Efg1p, leading to the expression of Tec1p. Tec1p and Cph2p then synergistically regulate medium-dependent, germ tube specific genes (Lane *et al.*, 2001b). The ligand(s) that induce this pathway are still unknown, as are the receptors or upstream effectors of Cph2p.

1.4.2 Repressors of Germ Tube Formation

Repression of germ tube formation in *C. albicans* is mediated by many environmental factors. Conditions that downregulate germ tube formation in liquid media are low pH, low temperature, high cell density, and in some conditions, high glucose concentrations. On solid media, high osmolarity also inhibits germ tube formation. *In vivo* inhibition of germ tube formation is mediated by gamma-interferon when lymphocytes contact invading cells (Kalo-Klein and Witkin, 1990), and some antifungal drugs such as amphotericin and azole antifungals inhibit germ tube formation by as yet unidentified mechanisms (Rogers *et al.*, 2003; Henry *et al.*, 1999). Pathways that negatively regulate germ tube formation in *C. albicans* are only just being identified and the ligands responsible for their activation and most other components of the pathway are still unknown.

1.4.2.1 The Tup1-mediated pathway

Tup1p and Ssn6p form a complex that represses transcription of germ tube specific genes (Jabet *et al.*, 2000). The N-terminal domain of Tup1p mediates multimerisation of Tup1p, and associates with a tetratricopeptide repeat (TPR) motif found in Ssn6p, creating a core complex containing three Tup1p molecules and one Ssn6p molecules (Jabet *et al.*, 2000). Regulation of the Tup1p/Ssn6p complex is mediated by different classes of DNA binding proteins, most likely transcription factors. They can bind directly to the individual Tup1p components via seven WD40 repeats found in their N-termini, or to the outer surface of the TPR domain found in Ssn6p. The interaction

of specific DNA binding proteins to the Tup1p/Ssn6p complex enables it to repress different subsets of germ tube specific genes, by binding to their promoters indirectly through the specific DNA binding proteins (Braun *et al.*, 2000; Murad *et al.*, 2001b). The repression then arises by either direct interaction with the polymerase II holoenzyme (Lee *et al.*, 2000), or by alteration in the local chromatin structure (Edmondson *et al.*, 1996). One DNA binding protein, (Nrg1p) has been identified. Nrg1p is a zinc finger DNA binding protein that interacts specifically with promoters that contain a C_4T sequence. Transcription analysis of Tup1p regulated genes shows that Nrg1p represses only a subset of Tup1p regulated genes, suggesting other regulators do exist (Murad *et al.*, 2001a). How Tup1p itself is regulated by the signal for repression is also unknown. Regulation could occur through direct activation of Tup1p or, more likely, through the DNA binding proteins, as is proposed with Nrg1p (Braun *et al.*, 2001).

1.4.2.2 The Rbf1-mediated pathway

The Rbf1p pathway is the least understood, and to date the only component identified, is the transcription factor Rbf1p. Rbf1p is a member of the bHLH family of transcription factors and seems to act in a general germ tube repression pathway independent of Tup1p (Ishii *et al.*, 1997; Sharkey *et al.*, 1999). Complicating the identification of potential upstream regulators of the Rbf1p pathway is the fact that no known fungal orthologs to Rbf1p are known.

1.5 Inducers of germ tube formation

Germ tube formation is triggered by a wide range of inducers such as, *N*-acetyl glucosamine, proline, complex media such as Lee's and Soll's, and serum (Mattia *et al.*, 1982, Leberer *et al.*, 2001). Serum is the most potent inducer of germ tube formation and remains the medium of choice for rapid identification of *C. albicans* from other non-*albicans Candida* species. Using *C. albicans* strain A72 it has recently been shown that glucose is the only dialyzable inducer present in serum (Hudson and Farley, unpublished). It has been postulated that glucose and other inducers bind to surface receptors, which then transduce the signal for germ tube formation. So far only one receptor has been shown to be involved in germ tube induction in *C. albicans*, Cys1p. Cys1p is an amino acid sensor, which is required for efficient germ

tube formation on solid media that contain amino acids such as Lee's and YPD serum (Brega *et al.*, 2004). Solid media such as *N*-acetyl glucosamine, Spider, and SLAD, which do not contain amino acids, are still able to induce germ tube formation by an as yet, unidentified biochemical pathway, as are liquid Lee's and serum-based media. Analysis of the *C. albicans* genome has identified orthologs to other nutrient receptors in *S. cerevisiae*, such as *SNF3* and *RGT2* that may also be involved in germ tube formation (Fan *et al.*, 2002).

1.6 Aims of this study

The hypothesis formulated for this study was that clinical isolates of *C. albicans* can, like strain A72, be induced to form germ tubes by buffered glucose, and that this induction signal is communicated by a glucose surface receptor. The aims of this study are:

- To test a panel of randomly selected *C. albicans* clinical isolates for germ tube formation in selected serum fractions and buffered glucose.
- To evaluate, by creating homozygous deletion mutants and analysis of their phenotype, two candidate glucose receptors and a glucose transporter for their role in germ tube formation in *C. albicans*.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Yeast strains

Yeast strains used in this study are listed in Tables 1 and 2, and were cultured as outlined in Section 2.3.1.

2.2 Media and Buffers

2.2.1 Media

Unless stated otherwise, all media were sterilised by autoclaving at 121°C for 15 min. Media were prepared with MilliQ water. Solid media was cooled to 65°C before pouring plates. Sterilised, uninoculated liquid media was cooled and stored at room temperature before use. Uninoculated plates were stored at 4°C. Where required supplements were added at the following concentrations, histidine (20 mg/l) arginine (20 mg/l), and uridine (80 mg/l).

Biotin Salts Agar

Biotin salts agar consisted of, per litre, 0.5 g $(NH_4)_2SO_4$, 0.5 g $CaCl.2H_2O$, 0.05 g $MgSO_4.7H_2O$, 2.0 g KH_2PO_4 (pH 5.2), 10.0 g glucose, 15 g bacteriological agar, and 25 µg of filter sterilised biotin. All components, except the biotin and KH_2PO_4 (pH 5.2), were dissolved in water, the pH adjusted to 5.2 and autoclaved. Biotin and KH_2PO_4 (pH 5.2) were added before pouring plates. In some experiments the glucose concentration was altered as stated, and in other experiments, other carbon sources (10.0 g/l) were substituted for glucose.

Dialysed Serum

Bovine serum (50 ml) was dialysed against 10 changes, each of 2 L for two hours, of 0.8% NaCl, in 10K-cutoff dialysis tubing and then stored at -20°C.

Table 1 Yeast strains used for analysis of germ tube formation

Strain	Relevant Characteristics	Cluster Strain	Source	Reference
A72	Wild-type *C. albicans* strain	No		Clinical isolate
IR40	Protease deficient strain derived from Sc5314, parent to BWP17.	No		
SA310	Wild-type *C. albicans* strain	No		Clincal isolate
3153a	Undergoes phenotypic switching on non-assimilative carbon sources	No		Vargas *et al*., (1994)
Gay-mc	Wild-type *C. albicans* strain	No	Oral	Schmid., *et al* (1999)
CH3	Wild-type *C. albicans* strain	No	Catheder	Schmid., *et al* (1999)
OTG10	Wild-type *C. albicans* strain	No	Nappy	Schmid., *et al* (1999)
HUN61	Wild-type *C. albicans* strain	No	Mouth	Schmid., *et al* (1999)
HUN68	Wild-type *C. albicans* strain	No	Mouth	Schmid., *et al* (1999)
CLB42	Wild-type *C. albicans* strain	Yes	Vaginal	Schmid., *et al* (1999)
Ysu568	Wild-type *C. albicans* strain	Yes	Urine	Schmid., *et al* (1999)
Sc5314	Strain used in *C. albicans* genome sequencing project	Yes		Clinical isolate
OD8807	Wild-type *C. albicans* strain	Yes	Oral	Schmid., *et al* (1999)
HUN96	Wild-type *C. albicans* strain	Yes	Blood	Schmid., *et al* (1999)
RIHO10	Wild-type *C. albicans* strain	Yes	Blood	Schmid., *et al* (1999)
FJ23	Wild-type *C. albicans* strain	Yes	Urine	Schmid., *et al* (1999)
FJ26	Wild-type *C. albicans* strain	Yes	Pus	Schmid., *et al* (1999)

Table 2 Yeast strains used for genetic analysis

#Strain	Relevant Characteriistics	Source	Reference
BWP17	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *arg4::hisG/arg4::hisG*	Dr. Aaron Mitchell	Enloe *et al.*, (2000)
BGT1a	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *orf19.1944::UAU1/ORF19.1944*	This study	
BGT1b	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *orf19.1944::UAU1/ORF19.1944*	This study	
TGT1a	*orf19.1944::UAU1/orf19.1944::URA3/ORF19.1944*	This study	
TGT1b	*orf19.1944::UAU1/orf19.1944::URA3/ORF19.1944*	This study	
DGT1c	*orf19.1944::UAU1/orf19.1944::URA3*	This study	
DGT1d	*orf19.1944::UAU1/orf19.1944::URA3*	This study	
BWA1a	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *orf19.5962::UAU1/ORF19.5962*	This study	
BWA1b	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *orf19.5962::UAU1/ORF19.5962*	This study	
BWA1c	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *orf19.5962::UAU1/ORF19.5962*	This study	
TWA1a	*orf19.5962::UAU1/orf19.5962::URA3/ORF19.5962*	This study	
TWA1b	*orf19.5962::UAU1/orf19.5962::URA3/ORF19.5962*	This study	
DWA1a	*orf19.5962::UAU1/orf19.5962::URA3*	This study	
DWA1b	*orf19.5962::UAU1/orf19.5962::URA3*		
*BGR1a	*ura3*Δ::λ*imm434/ura3*Δ::λ*imm434* *orf19.3668::UAU1/ORF19.3668*	This study	
*DGR1a	*orf19.3668::UAU1/orf19.3668::URA3*	This study	
*DGR1b	*orf19.3668::UAU1/orf19.3668::URA3*	This study	

#All strains carry the additional mutation *his1::hisG/his1::hisG*

*Deletion extends an unknown distance beyond the 5'end of orf19.3668

Filter Sterilised Serum

Bovine serum was passed twice through a cotton wool Millipore® prefilter AP25, twice through a 0.8 µm pore Whatman™ GF/A glass microfibre filter, twice through a 0.45 µm Millipore MF™ HA membrane filter and once through a 0.2 µm Pall™ GH Polypro filter. Filtration was carried out at 4°C. Filter sterilised serum was stored in 1 ml aliquots at -20°C.

Lee's Broth

Lee's broth, per litre, contained 0.5 g L-alanine, 1.3 g L-leucine, 1.0 g L-lysine, 0.1 g L-methionine, 0.0714 g L-ornithine, 0.5 g L-phenylalanine, 0.5 g L-proline, 0.5 g L-threonine, 12.5 g glucose, 5.0 g $(NH_4)_2SO_4$, 0.2 g $MgSO_47H_2O$, 5.0 g NaCl, 2.5 g K_2HPO_4 (anhydrous), and 0.001 g biotin. All components, except the K_2HPO_4 and biotin were dissolved in MilliQ water, and autoclaved. The broth was cooled to 65°C and the K_2HPO_4, and biotin, filter sterilised, added.

Serum Agar

Serum agar contained yeast extract (1% w/v), peptone (2% w/v), serum (10% w/v), and bacteriological agar (1.5% w/v). Bovine serum was filter sterilized (Section 2.2.1) and added to media that had been autoclaved and then cooled to 55°C.

Serum Filtrate (F1)

Bovine serum was diluted 5 fold in MilliQ water and diafiltered using a 10K-cutoff membrane. The final dilution was 12.5 fold and therefore 50 ml aliquots of the filtrate were freeze dried and resuspended in 4 ml MilliQ water before use. The filtrate was stored at -20°C.

Spider Agar and Broth (Leberer *et al.*, 1996)

Spider agar contained Nutrient Broth (1% w/v), K_2HPO_4 (0.2% w/v), Glucose (1% w/v) and bacteriological agar (2% w/v). The pH of the media was adjusted to 7.2 before the addition of the agar and amino acids, and sterilised by autoclaving. Spider broth was prepared by omitting the bacteriological agar.

Synthetic Low Ammonia Dextrose (SLAD) Agar (Lorenz and Heitman, 1997)

SLAD medium contained Yeast Nitrogen Base, without amino acids or ammonium sulphate (0.17% w/v), glucose (2% w/v), $(NH_4)_2SO_4$ (50μM), and bacteriological agar (2% w/v). The medium was sterilised by autoclaving.

Yeast Extract, Peptone and Dextrose (YPD) broth and YPD agar (Lo *et al.*, 1997)

YPD agar contained yeast extract (1% w/v), peptone (2% w/v), glucose (2% w/v) and bacteriological agar (2% w/v). YPD broth was prepared by omitting the bacteriological agar. In some experiments other carbon sources (2% w/v) were substituted for glucose.

Yeast Extract, Peptone and Dextrose (YPD), serum agar (Lane *et al.*, 2001a)

YPD serum agar was YPD agar containing 10% (w/v) serum. The media was autoclaved and then cooled to 65°C before the addition of filter sterilised bovine serum.

Yeast Nitrogen Base (YNB) agar

YNB agar contained Yeast Nitrogen Base, without amino acids or ammonium sulphate (0.67% w/v), ammonium sulphate (0.5% w/v), dextrose (2% w/v) and bacteriological agar (2% w/v). The medium was sterilised by autoclaving. In some experiments other carbon sources (2% w/v) were substituted for glucose.

2.2.2 Buffers and Solutions

Buffers and solutions requiring sterilisation were autoclaved at 121°C for 15 mins or passed through a 0.22 µm filter. MilliQ water was used in all instances. All buffers and solutions were stored at room temperature unless stated otherwise.

DNA loading dye

DNA loading dye was 80% (w/v) sucrose, 6 mM EDTA and 6% (w/v) bromophenol blue.

EDS solution

EDS solution was 50 mM EDTA, pH 8.5 containing 0.2% SDS.

PLATE buffer

PLATE buffer was 10 mM Tris/HCl, pH 7.5 containing 1 mM EDTA, 0.1 M lithium acetate and 40% (w/v) PEG 3350.

100x Salt Stocks

Stocks solutions of $MgSO_4$ (1.0 M), $CaCl_2$ (400 mM) and $MnSO_4$ (140 mM) were autoclaved and then mixed and diluted with sterile MilliQ water to give a final concentration of 120 mM $MgSO_4$, 4 mM $CaCl_2$, and 1.4 mM $MnSO_4$. This was then stored in 1 ml aliquots at 4°C.

SEB solution

SEB solution was 1 M sorbitol containing 0.1 M EDTA, pH 7.5 and 14 mM β-mercaptoethanol.

50x TAE

Used for agarose gel electrophoresis, 50x TAE contained 50mM Tris, 0.11% (v/v) glacial acetic acid, and 1 mM EDTA. The pH was adjusted to pH 8.0 with HCl or NaOH.

10x TE buffer

10x TE buffer was 10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA.

TELiOAc buffer

TELiOAc buffer was 10 mM Tris-HCl, pH 7.5 containing 1 mM EDTA and 0.1 M lithium acetate.

2.3 Bacteriological Methods

2.3.1 Culture conditions

All wild-type *C. albicans* strains were routinely cultured on YPD plates (Section 2.2.1). Strain Bwp17 and derivatives were routinely cultured on YNB plates (Section 2.2.1). Plates were placed in a 30°C incubator for 1 to 5 days. Single colonies were used to inoculate all solid and liquid media.

2.3.2 Storage of strains

For long term storage of yeast strains, sterile glycerol was added to 1 ml of an overnight liquid culture to give a final concentration of 20% (v/v). The glycerol stocks were stored at -80°C until required. Aliquots were revived by plating an aliquot of the glycerol stock on YPD or YNB agar. Short-term storage of yeast strains was on agar plates at 4°C.

2.3.3 Determination of *C. albicans* cell numbers

For each *C. albicans* strain tested a standard curve was prepared using an overnight culture of cells and a Pharmacia-Biotech Ultrospec®200 Spectrophotometer (Appendix 1).

2.4 *Candida albicans* Germ Tube Induction Assays

2.4.1 Liquid Assays

Assays were performed in a sterile 96-well microtitre plate. Prior to the addition of cells to the incubation mixture, the plate was covered with a plate sealer and pre-incubated at 37°C with shaking at 250 rpm. Cells were harvested from 50µl of a 3 ml overnight culture in YPD broth (Section 2.2.2) by centrifugation at 15,000 rpm for 5 minutes. The supernatant was removed and the cells were washed twice in 500 µl of pre-warmed 37°C MilliQ water. Washed cells (2 µl) were then added to each germ tube induction assay. The plate was incubated at 37°C with shaking at 250 rpm for 2 hours. Cells and germ tubes were then counted using an inverse microscope. All experiments included negative and positive controls, consisting of MilliQ water and 10% serum, respectively.

2.4.2 Solid Assays

Induction of germ tubes on YPD serum, SLAD and spider agar (Section 2.2.1) was performed by inoculating the appropriate plates with single colonies, using sterile toothpicks. The plates were sealed with parafilm and incubated at 37°C for 10 – 14 days. The embedded agar assay was performed by inoculating a YPD plate with five single colony stabs. The plate was incubated for 24 hrs at 28°C and then overlaid with 20 ml of YPD agar, followed by a further incubation at 22°C for 7 days. The formation of hyphae was initially assessed using a light microscope, but in the later stages of the incubation hyphal growth was visible without the aid of a microscope.

2.5 DNA manipulation

2.5.1 Purification of genomic DNA from yeast strains

The method used for purifying *C. albicans* genomic DNA was based on that of McEachern and Hicks (1993). YPD cultures (3 ml) were grown overnight and the cells harvested by centrifugation at 13000 rpm for 5 min. The cell pellet was resuspended in 0.5 ml of SEB (Section 2.2.2), and (100 µl) 100T Zymolyase (1mg/ml) and incubated with regular mixing, for 2 hrs at 37°C. The spheroplasts were centrifuged at 13000 rpm for 5 min, resuspended in 0.5ml of EDS (Section 2.2.2), proteinase K (50µg) was added and the suspension was incubated at 37°C for 2 hrs. The suspension was then heated to 65°C for 15 min, mixed with 50 µl of 3.0 M potassium acetate (pH 5.2), and stored on ice for 1 hour. The suspension was then centrifuged at 13000 rpm for 5 min and an equal volume of isopropanol was added to the supernatant before centrifugation at 13000 rpm for 10 mins. The genomic DNA pellet was washed with ethanol and dissolved in 100 µl of TE (Section 2.2.2) and extracted once with an equal volume of phenol and once with an equal volume of chloroform. DNA was precipitated from the aqueous phase with 1 volume of absolute ethanol, washed with 70% ethanol, air-dried and dissolved in 30 µl of TE containing 1 µl of RNase A (10 mg/ml), to digest ribosomal RNA, and incubated at room temperature overnight. The DNA was then stored at -20°C.

2.5.2 Agarose gel electrophoresis of DNA

PCR products and total genomic DNA were analysed by electrophoresis at 80- 110V for 1.5 to 2 hrs, through 0.8% agarose gels, prepared in 1 x TAE buffer (Section 2.2.2). Agarose gels were stained with ethidium bromide for 15 min and destained for no less than 10 mins in distilled water before being visualised using a UV Transilluminator. Gels were photographed with a Heliopan S49 camera. The 1 kb^+ DNA ladder was used as a size standard.

2.5.3 Quantitation of DNA

Genomic DNA was quantitated using absorbance at 260nm. PCR products were quantitated using agarose gel electrophoresis as outlined in Section 2.5.2, and 5-20 ng DNA standards.

2.6 PCR amplification of DNA

2.6.1 Oligonucleotide primer design

Sequence of the *C. albicans* genome was obtained from the Stanford genome website, http://www-sequence.stanford.edu/group/candida/. Oligonucleotide primers were designed from *C. albicans* genome contiguous (contig) sequences using the computer program Amplify, and the internet based program, Web Primer (http://seq.yeastgenome.org/cgi-bin/SGD/web-primer). Primers were purchased from Sigma® Genosys (Sigma-Aldrich) Australia. Oligonucleotide primers used in this study are listed in Tables 3, 4, 5 and 6.

Table 3 orf19.1944 oligonucleotide primers used in this study

orf19.1944 Primers		
Primer Name	Oligonucleotide Sequence (5' to 3')	Primer Function
orf19.1944_5'DR	AGCCTGTCCTTACAGTCTATTGTCCAATATCATTAATAATCA TTCACATAATATCGAACTGTTTTCCCAGTCACGACGTT	Mutagenesis
orf19.1944_3DR	TATCATTATCCACAGTATGTAATTCATCGATATCTTCATTG GATTTTACTCTATCAATTGGTGGAATTGTGAGCGGATA	Mutagenesis
orf19.1944_amp3	CCAATTCTCCATCATCATTATCATC	Genotyping
orf19.1944_amp5	AAAAGCACCCTATTTTCCTTACAAG	Genotyping
orf19.1944_5amp2	CAATTTCAATTTTATTTACCCCCAAG	Genotyping
1944INTPRIMER	CTTTCCCGACTAACATTGCTGAG	Genotyping

Table 4 orf19.3668 oligonucleotide primers used in this study

orf19.3668 Primers		
Primer Name	Oligonucleotide Sequence (5' to 3')	Primer Function
PCF_3668_5DR	GTAGAAGAAAATCCGGGGAGGTGGAGAGGAAAAAGCC ATTCTGCTATGTTTCGTGCTGCAAGGCGATTAAGT	Mutagenesis
3668_3DR	TTTGAAAATAACATAAAAGATATTATAAAATAACAAATTTAA AATTAGAGAATTAAAAGAAGTGGAATTGTGAGCGGATA	Mutagenesis
orf3668_3amp	GAACGAAGCTATACAGGAATCATAA	Genotyping
orf3668_5amp	GATGATATACATATAACGCAGACAC	Genotyping
orf3668_5ampB	CGAAAAGCCAAAAAAATACGCAC	Genotyping
orf3668_5amp3	AATTTTTTGGGGTTATTGGGGG	Genotyping
intPrime3668	CGGTCTTGCTGGTATCTTAGG	Genotyping

Table 5 orf19.5962 oligonucleotide primers used in this study

orf19.5962 Primers		
Primer Name	Oligonucleotide Sequence (5' to 3')	Primer Function
orf5962_5'DR	TTTTTAACTCGTATAATGTACGATGCTTCATTGGAAGA CGAGTATTATCGTCAAACTGCAAGGCGATTAAGTTGGGTAAC	Mutagenesis
orf5962_3'DR	TTCATCATCACTCGAATCTGAATCACTTGGAGGATGAG CAAAAAATGGTGTTGCTATCACTGTGGAATTGTGAGCGGATA	Mutagenesis
orf5962_amp3	CAACCTCTATATGACACCCTTATTT	Genotyping
orf5962_amp5	CTTAATTCGATCACATTCAAGTGTT	Genotyping
5962intPrime	GCCATATTTGTCTTTTTCACCGT	Genotyping

Table 6 Common oligonucleotide primers used in this study

Common Primers		
Primer Name	Oligonucleotide Sequence (5' to 3')	Primer Function
Ygr189amp3	GAACTGCATTGGATTTTCGTC	PCR control
Ygr189amp5	CTACTACTTATGATCGTGGT	PCR control
Arg4Det	GGAATTGATCAATTATCTTTTGAAC	Genotyping
5Arg4Det	CAAAAGTACACGACCCACAGTTAGTC	Genotyping
UAU_ComSeq	TACGAATCAATGGCACTACAGCAAC	Sequencing
5UAUComSeq	GGGGGAGATTTTCACTTTATTAG	Sequencing
3-detect	TGTGGAATTGTGAGCGGATAACAATTTCAC	Genotyping
5-detect	GTTTTCCCAGTCACGACGTTGTAAAACGAC	Genotyping

2.6.2 PCR amplification of DNA targets

PCR amplification was performed using a PTC-200 Peltier Thermal Cycler (MU Research) or a Mastercycler® Gradient Thermal Cycler (Eppendorf). PCR reactions contained 2.5–5 ng/10µl of *C. albicans* genomic DNA or 5 ng/10µl of plasmid pBME101 DNA, 10 pmol/10 µl of each primer, 10 mM Tris-HCl, 5 mM KCl pH 8.3, 2.5 mM $MgCl_2$, 0.2 mM dGTP, dCTP, dATP, dTTP, and 0.2 units/10 µl *Taq* polymerase. Four different thermocycling protocols were used. Amplification of DNA for transformation of *C. albicans* strain Bwp17 began with an initial denaturation step of 94°C for 1 min. Followed by 31 cycles of a denaturation step (94°C for 1 min), an annealing step (50°C for 1 min) and an elongation step (72°C for 4.5 min). Amplification of the wild-type ORF and the URA3 insert from genomic DNA began with an initial denaturation step of 94°C for 1 min. Followed by 31 cycles of a denaturation step (94°C for 1 min), an annealing step (57°C for 1 min) and an elongation step (72°C for 4 min). Amplification of the 3' or 5' end of the *UAU1* insertion began with an initial denaturation step of 94°C for 1 min. Followed by 31 cycles of a denaturation step (94°C for 1 min), an annealing step (55°C for 1 min) and an elongation step (72°C for 2 min). Amplification of the internal region of the wild-type ORFs began with an initial denaturation step of 94°C for 1 min. Followed by 31 cycles of a denaturation step (94°C for 1 min), an annealing step (55°C for 1 min) and an elongation step (72°C for 2 min). All reactions were subjected to an additional elongation step of 72°C for 10 min to ensure that all products were full length. Negative controls containing all reagents except template were performed. For each new primer a single primer PCR reaction was performed to ensure that PCR products were specific to reactions containing both forward and reverse primers.

2.6.3 Purification of PCR products

Purification of PCR products was carried out using (QIAGEN™ Easy Prep PCR Product prep kit). Purified PCR products were quantitated following electrophoresis as outlined in Section 2.5.3.

2.6.4 Sequencing of PCR products

PCR products were sequenced using a BigDye™ Terminator Version 3.1 Ready Reaction Cycle Sequencing kit and analysed by a capillary ABI3730 Genetic

Analyzer (Applied Biosystems Inc.) at the Massey University Sequencing Service,. This kit uses the dideoxy chain termination method (Sanger *et al.*, 1977).

2.6.5 Preparation of PCR products for use in transformation

PCR products from 100 µl reactions were transferred to sterile 1.5 ml eppendorf tubes and 30 µl of 7.5 M ammonium acetate and 250 µl of ethanol added. The mixture was incubated for 30 min at -20°C, spun for 5 min at 15, 000 rpm, washed with 200 µl ice cold 70% ethanol and dried at 37°C for 15 min. The pellet was then dissolved, on ice, in 5µl of 1x TE pH 8.0 (Section 2.2.2) for 30 – 60 min.

2.7 Disruption of target ORFs

2.7.1 Transformation of *C. albicans* strain Bwp17

A 50 ml YPD culture was inoculated with 0.5 ml of a 2 ml overnight YPD culture and incubated at 28°C with shaking at 250 rpm until the OD_{600} of the culture had increased 4. If the OD_{600} had not increased 4 fold in 6 hours, the culture was discarded. Cells were pelleted at 15, 000 rpm for 10 mins, washed once with 5 ml sterile MilliQ water and resuspended in 0.5 ml TELiOAc solution (Section 2.2.2). A portion (0.1 ml) of the cell suspension was transferred to previously prepared PCR products (Section 2.6.5) and incubated at room temperature for 30 mins. Then 0.7 ml of PLATE buffer (Section 2.2.2) was added and the mixture incubated at room temperature for 16 hrs. Tubes then heat shocked at 44°C for 15 mins and cells pelleted at 15,000 rpm for 30 secs. The cells were resuspended in 0.1 ml sterile MilliQ water, spread on heterozygote selection YNB plates (Section 2.2.1) and incubated at 28°C for 3-4 days.

Homozygotes were generated by growing heterozygote colonies in 3 ml YP broth containing either, mannitol (2% w/v), xylose (2% w/v), sucrose (2% w/v) or glucose (0.2%, 2% 20% w/v) for 24 – 36 hrs (Section 2.2.1). The cells were recovered by centrifugation at 15,000 rpm for 30 secs, and resuspended in 300 µl sterile MilliQ water. Aliquots of 50 µl were plated onto homozygote selection YNB plates (Section 2.2.1) and incubated for 4-5 days at 28°C.

2.7.2 Screening of transformants

Arg^+ transformants that grew on the YNB heterozygote selection plates (Section 2.2.1) were single colony purified, twice, on the same medium. DNA extracted from these cells was analysed by PCR, to detect correct insertion of the *UAU1* cassette into the target ORF. The PCR contained the Arg4Det primer, which anneals within the cassette and a primer (named after the target ORF, with the suffix 3amp or amp3 added) complementary to the 3' region flanking the targeted disruption. Deletion of the target ORF was then confirmed using the 5Arg4Det primer, which anneals within the *UAU1* cassette and a primer (named after the target ORF, with the suffix 5amp or amp5 added), complementary to the 5'-region flanking the targeted disruption. Both PCR products were then sequenced to confirm the site of the deletion.

Following homozygote selection (Section 2.7.1), DNA from Arg^+/Ura^+ colonies was extracted and the presence of the *UAU1* cassette detected by PCR as described above. The recombined URA3 gene was detected using the 5' amp and 3' amp primers that were complementary to regions flanking the target ORF.

2.8 Characterisation of target ORFs *in silico*

2.8.1 BLAST, sequence alignment and sequence conversion programs

DNA and protein sequences for predicted glucose transporters and receptors were obtained from the Stanford Genome Technology Center (http://www-sequence.stanford.edu/group/candida/), assembly 19 (diploid assembly). BLASTN was done through the NCBI website (http://www.ncbi.nlm.nih.gov/) to confirm putative gene assignments of chosen ORFs. Protein sequences were confirmed using EMBOSS Transeq (http://www.ebi.ac.uk/emboss/transeq/index.html). Multiple sequence alignments were done using CLUSTALW 1.82 (http://www.ebi.ac.uk/clustalw/index.html), whilst global and local pair wise alignments were done using the ALIGN program (http://www.ebi.ac.uk/emboss/align/index.html). All alignments were done using the PAM250 matrix.

2.8.2 Use of motif search engines

Searches for conserved domains in the selected gene targets were performed using CD-search v1.63 on the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml) and the CDD v1.63 – 16482 PSSMs database. Transmembrane spanning regions of the protein sequences were predicted using the TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/) and SOSUI (http://sosui.proteome.bio.tuat.ac.jp/sosuimenu0.html) servers.

CHAPTER THREE: RESULTS

3.1 Germ tube induction in clinical isolates of *C. albicans*

A panel of clinical isolates was chosen at random to determine if the conclusions drawn from the analysis of germ tube formation by *C. albicans* strain A72 in glucose and serum fractions, was valid for other *C. albicans* isolates. Each clinical isolate was assayed for germ tube induction using serum, serum fractions, urea and buffered glucose as outlined in Section 2.4.1.

3.1.1 Germ tube formation in dialysed serum

In dialysed serum (molecular mass, > 10 kDa) *C. albicans* strain A72 formed only low numbers of germ tubes (Hudson and Farley, unpublished). To test if this result was applicable to other strains of *C. albicans*, nine clinical isolates were assayed for germ tube formation in dialysed serum. Dialysed serum was tested at two different concentrations (10% and 50%) to determine whether any activity lost could be restored by simply increasing the concentration of the remaining components. Dialysed serum was less effective at inducing germ tube formation than serum (Table 7). The difference was statistically significant ($P < 0.05$, Students *t*-test) for all the clinical isolates assayed at equivalent concentrations of serum and dialysed serum. Even when the concentration of dialysed serum was increased five fold, this difference was still statistically significant ($P < 0.05$, Students *t*-test) for six of the nine clinical isolates. The three exceptions showed large variations in germ tube formation in serum between experiments. To test if the low molecular weight components, removed from serum by dialysis, could be replaced solely by glucose, exogenous glucose was added to the dialysed serum fractions. A comparison of the percentage germ tube formation in 10% dialysed serum supplemented with exogenous glucose, and serum, showed no statistically significant difference for seven of the nine clinical isolates tested (Table 7). Thus, the addition of just glucose to dialysed serum can restore induction of germ tube formation to levels seen in serum, in the majority of clinical isolates tested.

C. albicans Strains	Germ Tube Formation (%) in				
	Serum (10%)	Dialysed Serum (10%)	Dialysed Serum (50%)	Dialysed Serum (10%) with glucose	Dialysed Serum (50%) with glucose
A72	95 ± 5 (8)	23 ± 20 (5)#	50 ± 25 (5)#	82 ± 21 (5)	82 ± 20 (5)
Sc5314	92 ± 4 (3)	22 ± 10 (2)#	23 ± 11 (2)#	93 ± 1 (2)	96 ± 0 (2)
Gay-mc	85 ± 5 (4)	5 ± 3 (3)#	30 ± 5 (3)#	55 ± 29 (3)	64 ± 17 (3)
FJ26	82 ± 5 (3)	28 ± 13 (2)#	41 ± 16 (2)#	67 ± 4 (2)#	83 ± 8 (2)
SA310	75 ± 23 (3)	6 ± 3 (3)#	16 ± 22 (3)#	22 ± 12 (3)#	76 ± 16 (3)
HUN96	82 ± 14 (3)	21 ± 29 (2)#	24 ± 19 (2)#	29 ± 41 (2)	88 ± 4 (2)
RIHO10	62 ± 36 (4)	1 ± 1 (4)#	21 ± 14 (4)	21 ± 2 (4)	51 ± 30 (4)
OTG10	71 ± 5 (3)	5 ± 6 (2)#	21 ± 22 (2)#	31 ± 28 (2)	28 ± 3 (2)#
IR40	51 ± 37 (2)	0 ± 0 (2)#	6 ± 8 (2)	20 ± 5 (2)	33 ± 13 (2)
3153a	25 ± 30 (4)	1 ± 2 (4)#	3 ± 3 (4)	8 ± 7 (4)	20 ± 15 (4)

Table 7 Germ tube induction by dialysed serum

Cells were assayed for germ tube formation as outlined in Section 2.4.1, and the results presented as the average ± standard deviation for the number of experiments shown in parenthesis. Where glucose was present the final concentration was 0.5mM. Strain A72 was included as a control. No germ tube formation was observed in water only controls.

Statistically significantly different (Students *t*-test, $P < 0.05$) to the percentage germ tube formation in serum.

3.1.2 Germ tube formation in serum filtrate

Analysis of serum filtrate (molecular mass, < 10 kDa) mediated germ tube formation in *C. albicans* strain A72 showed that serum filtrate was able to induce germ tube percentages comparable to those of serum (Hudson and Farley, unpublished). To test if this result was applicable to other strains of *C. albicans*, twelve clinical isolates were assayed for germ tube formation in serum filtrate. As had been found for strain A72, serum filtrate was as effective as serum at inducing germ tubes in all the clinical isolates tested, except strain OTG10 (Table 8). The low level of germ tube induction for clinical isolate CLB42 under any of the test conditions suggested that it might not be a *C. albicans* isolate. DNA fingerprinting by Dr. Jan Schmid confirmed that it was a strain of *C. albicans* (Schmid *et al.*, 1999). Also, there was no evidence that the culture was a mixture of strains (five independently derived colonies from single cells were assayed and all produced about 4% germ tubes).

C. albicans Strains	Germ Tube Formation (%) In Serum	Germ Tube Formation (%) In Serum Filtrate
A72	95 ± 5 (8)	93 ± 4 (8)#
Sc5314	92 ± 4 (3)	65 ± 28 (3)#
Gay-mc	85 ± 5 (4)	79 ± 10 (4)#
HUN61	85 ± 4 (3)	82 ± 10 (3)#
FJ26	82 ± 5 (3)	80 ± 6 (3)#
SA310	75 ± 23 (3)	81 ± 19 (3)#
HUN96	82 ± 14 (3)	79 ± 15 (3)#
RIHO10	62 ± 36 (4)	37 ± 24 (4)#
OTG10	71 ± 5 (3)	55 ± 1 (3)
OD8807	37 ± 35 (3)	22 ± 22 (3)#
IR40	51 ± 37 (2)	22 ± 31 (2)#
3153a	25 ± 30 (4)	25 ± 20 (4)#
CLB42	4 ± 1 (2)	2 ± 1 (2)#

Table 8 Germ tube induction by serum filtrate

Cells were assayed for germ tube formation as outlined in Section 2.4.1. Germ tube formation in 10% serum, or serum filtrate equivalent to 10% serum are presented as the average value ± standard deviation for the number of experiments shown in parenthesis. Strain A72 was included as a control. No germ tube formation was observed in water only controls.

No statistically significant difference (Students *t*-test, $P > 0.05$) to the percentage germ tube formation in serum

3.1.3 Induction of germ tube formation by glucose

Previous research using strain A72 has shown that the only inducer present in the serum filtrate is glucose (Hudson and Farley, unpublished). To investigate if glucose is an inducer for other strains of *C. albicans*, a panel of sixteen clinical isolates was assayed using glucose buffered to the pH of serum. In addition, two controls were included; buffer alone, to assess germ tube formation in the absence of inducer, and glucose alone, to assess the requirement for pH 8.0. Buffered glucose was able to induce germ tubes in all the clinical isolates assayed, except strain CLB42, which was also a low responder in serum (Table 9). For ten out of the sixteen clinical isolates, the induction by buffered glucose was not statistically significantly different to serum ($P > 0.05$, Students *t*-test).

C. albicans Strains	Germ Tube Formation (%) In		
	Unbuffered glucose	Serum	Buffered glucose
A72	56 ± 19 (8)	95 ± 5 (8)	86 ± 6 (8)
Sc5314	53 ± 14 (3)#	92 ± 4 (3)	51 ± 1 (3)
Gay-mc	24 ± 10 (4)	85 ± 5 (4)	70 ± 11 (4)
HUN61	65 ± 19 (3)#	85 ± 4 (3)	79 ± 17 (3)*
FJ26	32 ± 9 (3)#	82 ± 5 (3)	45 ± 16 (3)
SA310	42 ± 14 (3)#	75 ± 23 (3)	47 ± 20 (3)*
FJ23	68 ± 6 (2)#	83 ± 2 (2)	69 ± 19 (2)*
HUN96	27 ± 24 (3)#	82 ± 14 (3)	43 ± 17 (3)
CH3	46 ± 33 (2)#	73 ± 5 (2)	71 ± 1 (2)*
RIHO10	17 ± 23 (4)#	62 ± 36 (4)	24 ± 24 (4)*
HUN68	36 ± 2 (2)	79 ± 13 (2)	59 ± 6 (2)*
OTG10	18 ± 27 (3)#	71 ± 5 (3)	36 ± 20 (3)
Ysu568	63 ± 21 (2)#	50 ± 43 (2)	68 ± 2 (2)*
OD8807	9 ± 15 (3)#	37 ± 35 (3)	18 ± 30 (3)*
IR40	14 ± 9 (2)#	51 ± 37 (2)	25 ± 31 (2)*
3153a	9 ± 16 (4)#	25 ± 30 (4)	23 ± 20 (4)*
CLB42	0 ± 0 (2)#	4 ± 1 (2)	0 ± 0 (2)

Table 9 Germ tube induction by glucose

Cells were assayed for germ tube formation as outlined in Section 2.4.1 using serum (10% v/v), buffered glucose (0.5mM glucose in 50mM BICINE pH 8.0), and unbuffered glucose (0.5mM glucose). A72 was included as a control. No germ tube formation was observed in water only controls. Except for OTG10 (4 ± 7 (3)) no strains formed germ tubes in buffer alone.

* No statistically significant difference to the percentage germ tube formation observed in serum (Students *t*-test, $P > 0.05$).

No statistically significant difference to the percentage germ tube formation observed in buffered glucose (Students *t*-test, $P > 0.05$).

Whereas with *C. albicans* strain A72 germ tube formation is higher in buffered glucose than in glucose alone ($P < 0.05$, Students *t*-test) this result was not observed for most of the clinical isolates. The only other strains that gave a statistically significant difference in germ tube formation between buffered and unbuffered glucose were Gay-mc and HUN68.

C. albicans Strains	Germ Tube Formation (%) In											
	Serum		Urea		Buffered urea		Unbuffered glucose		Unbuffered glucose and urea		Buffered glucose		Buffered glucose and urea	
A72	95 ± 5	(8)	0 ± 0	(5)	0 ± 0	(5)	56 ± 19	(8)	28 ± 32	(18)	86 ± 6	(8)	69 ± 20	(5)
Sc5314	92 ± 4	(3)	0 ± 0	(2)	1 ± 1	(2)	53 ± 14	(3)	42 ± 34	(5)	51 ± 1	(3)	59 ± 10	(2)
Gay-mc	85 ± 5	(4)	0 ± 0	(3)	0 ± 0	(3)	24 ± 10	(4)	44 ± 5	(3)	70 ± 11	(4)	44 ± 5	(3)
FJ26	82 ± 5	(3)	0 ± 0	(2)	6 ± 8	(2)	32 ± 9	(3)	16 ± 17	(5)	45 ± 16	(3)	25 ± 27	(2)
SA310	75 ± 23	(3)	0 ± 0	(3)	1 ± 2	(3)	42 ± 14	(3)	36 ± 18	(4)	47 ± 20	(3)	39 ± 21	(3)
HUN96	82 ± 14	(3)	0 ± 0	(2)	0 ± 0	(2)	27 ± 24	(3)	28 ± 28	(5)	43 ± 17	(3)	41 ± 49	(2)
RIHO10	62 ± 36	(4)	0 ± 0	(4)	0 ± 0	(4)	17 ± 23	(4)	14 ± 17	(4)	24 ± 24	(4)	14 ± 17	(4)
OTG10	71 ± 5	(3)	5 ± 7	(2)	4 ± 5	(2)	18 ± 27	(3)	2 ± 3	(5)	36 ± 20	(3)	5 ± 4	(2)
IR40	51 ± 37	(2)	0 ± 0	(2)	0 ± 0	(2)	14 ± 9	(2)	13 ± 7	(6)	25 ± 31	(2)	16 ± 9	(2)
3153a	25 ± 30	(4)	0 ± 0	(4)	0 ± 0	(4)	9 ± 16	(4)	8 ± 13	(4)	23 ± 20	(4)	8 ± 13	(4)
CLB42	4 ± 1	(2)	0 ± 0	(2)	0 ± 0	(2)	0 ± 0	(2)	0 ± 1	(4)	0 ± 0	(2)	1 ± 1	(2)

Table 10 Germ tube induction by urea

Cells were assayed for germ tube formation as outlined in Section 2.4.1 using serum (10% v/v), urea (0.03mg/ml), buffered urea (0.03mg/ml urea in 50mM BICINE pH 8.0), unbuffered glucose (0.5mM glucose), buffered glucose (0.5mM glucose in 50mM BICINE pH 8.0), and buffered urea with glucose (0.03mg/ml urea in 50mM BICINE pH 8.0 with 0.5mM glucose). No germ tube formation was observed in water only controls.

3.1.4 Induction of germ tube formation by urea

Urea is one of the major components of serum, and important for normal budding cell growth and germ tube formation (Limjindaporn *et al.*, 2003). Since dialysed serum was a poor inducer of germ tube formation, and buffered glucose does not induce germ tubes to the same extent as serum in some strains, it was decided to test the effect of added urea on glucose induced germ tube formation. Urea and buffered urea induced germ tube formation in some strains but the percentage germ tube formation was less than 10% in all cases (Table 10). In all strains tested urea had no statistically significant effect ($P > 0.05$, Students *t*-test) on germ tube induction by glucose in the presence or absence of buffer (Table 10).

Standard conditions for induction of germ tubes in *C. albicans* by serum or glucose are a temperature of 37°C and pH 8.0. In an attempt to identify cells that were unable to form germ tubes under these conditions cells were first grown overnight at 37°C. However, the results were extremely variable, and this attempt to find conditions that might be used to identify differentially expressed genes important for germ tube formation was abandoned. It was hypothesised that glucose induces germ tube formation via a receptor so the genome was analysed for genes encoding putative glucose receptors. In the model yeast *Saccharomyces cerevisiae* three glucose receptors have been characterised. The first two to be characterised were Sucrose Non-Fermenting 3 (*SNF3*) and Restores Glucose Transport 2 (*RGT2*). These have been shown to regulate high and low-affinity glucose transport, respectively (Bisson *et al.*, 1987 and Neigeborn *et al.*, 1984). Structurally, each has 12 transmembrane spanning domains, as found in the Hexose Transporter (*HXT*) family (Ozcan *et al.*, 1998). However, *SNF3* and *RGT2* differ from the *HXTs* in that each has a carboxy terminal extension that is necessary for the transmission of the glucose signal (Ozcan *et al.*, 1998) and neither actively transports glucose. There are at least 18 functionally characterised *HXTs* in *S. cerevisiae* (Wieczorke *et al.*, 1999). A strain carrying a concurrent knockout of all 18 HXTs is unable to uptake glucose, yet how the *HXTs* are regulated by *SNF3* and *RGT2* is still to be determined. The most recent glucose receptor to be characterised in *S. cerevisiae* was G-protein coupled receptor 1 (*GPR1*). *GPR1* was identified in a yeast two-hybrid screen for proteins that interacted with the G-protein *GPA2*, which has been shown to be involved in glucose induction of the cAMP pathway and pseudohyphal differentiation (Kraakman *et al.*, 1999 and Lorenz *et al.*, 2000). An ortholog to *GPA2* has also been characterised in *C. albicans*. A *C. albicans* mutant carrying a deletion of *GPA2* is unable to undergo filamentation on SLAD and Spider medium and in embedded conditions, but still able to form germ tubes in media containing serum (Sanchez-Martinez and Perez-Martin, 2002). A notable difference between *GPA2* in *S. cerevisiae* and *C. albicans* is that whilst *S. cerevisiae GPA2* feeds into the cAMP pathway, *C. albicans GPA2* feeds into the mitogen activated protein kinase (MAPK) pathway (Sanchez-Martinez and Perez-Martin, 2002).

3.2 Characterisation of orf19.1944

Orf19.1944 was chosen based on its homology to the G-protein coupled-receptor 1 (*GPR1*) from *Saccharomyces cerevisiae* (Kraakman *et al.*, 1999; Lorenz., *et al.*, 2000). The putative gene product was characterised *in silico* (Section 2.8). A strain carrying a homozygous deletion of orf19.1944 was created and its ability to form germ tubes determined (Section 2.4.1).

3.2.1 *in silico* characterisation of orf19.1944

Orf19.1944 encodes a predicted 823 residue protein. The BLASTP program (Section 2.8.1) was used to assign a putative function to this gene product by comparing the amino acid sequence to a database of fungal genome DNA sequences. Hits with BLASTP were considered to be significant if the Expect (E) value was lower than 1e-10. Two of the hits were to *GPR1* from *S. cerevisiae* whilst one was to *GIT3*, a G-protein coupled receptor found in *Schizosaccharomyces pombe* (Welton *et al.*, 2000). These three annotations were all based on experimental evidence. The other top scoring hits were all theoretical proteins that had been assigned a putative G-protein coupled receptor function through sequence similarity. See Appendix 2 for the BLASTP results. The BLASTN program (Section 2.8.1) was also utilised to compare orf19.1944 and *GPR1* against the *C. albicans* genome to identify if any other ORFs with high homology were present. For orf19.1944 only itself was identified with an E-value higher than 1e-10 (Results not shown). For *GPR1* nineteen putative ORFs were identified with E-values higher than 1e-10 (Results not shown). All but one ORF encoded putative proteins of less than 35 amino acids. The high degree of similarity in the BLASTN search for these ORFs was due to the presence of nucleotide stretches that encoded a long polyasparagine region, which is found in *GPR1*. Contig19-10137 and its allelic counterpart Contig19-20137 contained the only ORFs (orf19.1944 and orf19.9499) that encoded a protein with sequence similarity and comparable length to *GPR1* from *S. cerevisiae* (Data not shown). The proteins encoded by these two ORFs are identical.

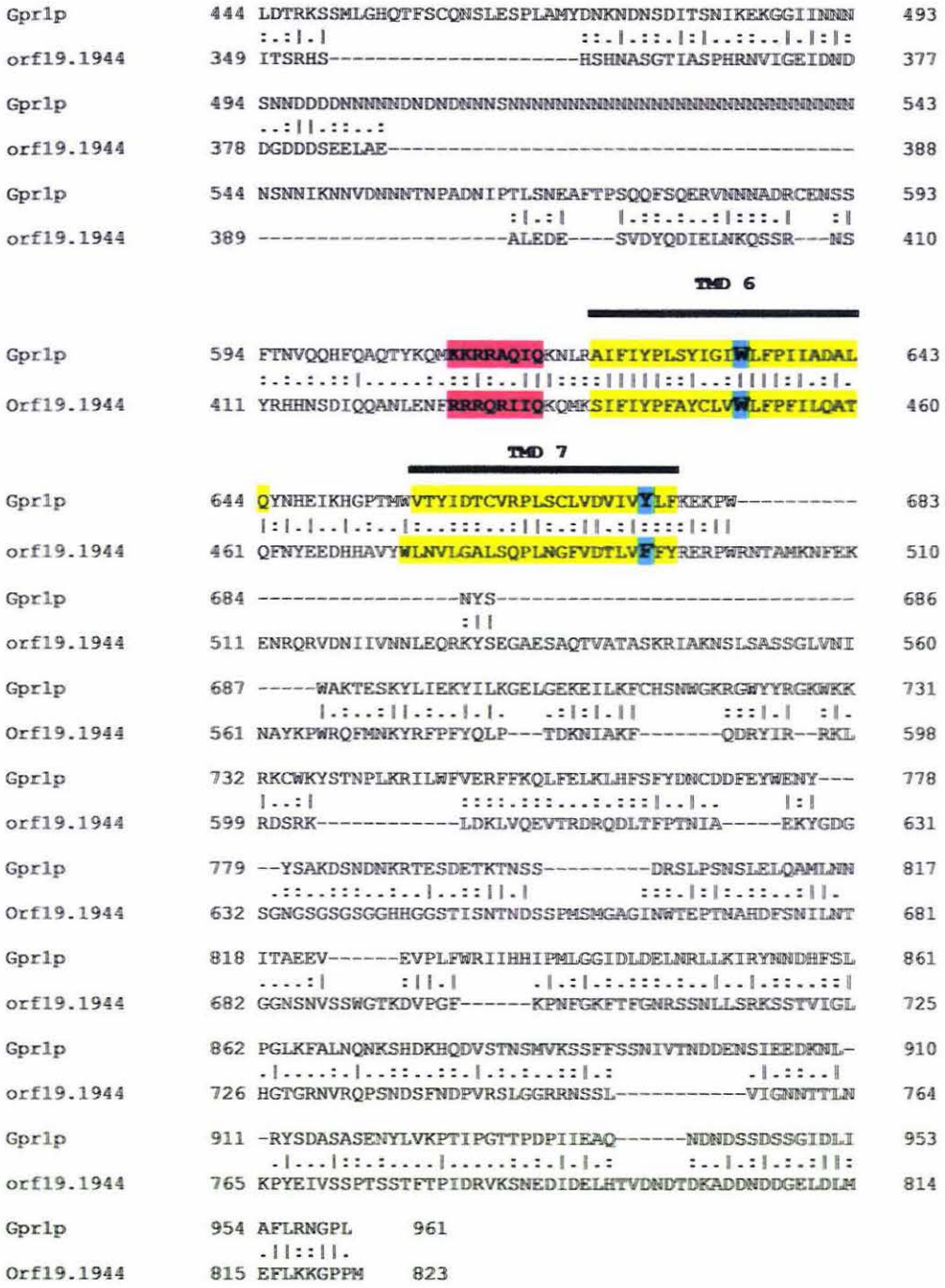


Figure 3 Global alignment of the protein encoded by orf19.1944 against Gprlp

Gprlp is the G-protein coupled receptor (GPCR) protein found in *Saccharomyces cerevisiae*. Highlighted in yellow are the predicted transmembrane domains (TMD) from the protein encoded by orf19.1944 and Gprlp. Highlighted in teal are known conserved amino acids found in GPCRs, and highlighted in red are basic stretches of amino acids found to be important for the function Gprlp and pheromone receptors in *S. cerevisiae*, one of which is present in the protein encoded by orf19.1944.

Both rpsBLAST and TMHMM were used to search for conserved motifs in the predicted orf19.1944 protein sequence as outlined in Section 2.8.2. A conserved domain search using rpsBLAST revealed no matches. TMHMM identified seven putative transmembrane domains (Figure 3), a characteristic of G protein-coupled receptors (Gether, 2000). The putative structure of this protein indicated that it contained a very large third cytoplasmic loop, between transmembrane domains five and six, of 153 amino acids and a long C-terminal cytoplasmic tail of 328 amino acids. Both are characteristic of G protein-coupled receptors. The protein encoded by orf19.1944 also contained the four amino acid residues present in the transmembrane domains of the G-protein coupled receptor superfamily. These are the alanine at position 236 in transmembrane domain 4, the phenylalanine at position 279 in transmembrane domain 5, the tryptophan at position 451 in transmembrane domain 6, and an aromatic residue at position 494 in transmembrane domain 7 (Figure 3). In the predicted arrangement of the receptor transmembrane helices (Gether, 2000), these residues all face away from the surrounding membrane lipid and toward the centre of the molecule or the other helices. Intramolecular interactions between these transmembrane α-helices are thought to maintain the structure of the receptor in the membrane and allow it to bind the G protein. These features of the protein encoded by orf19.1944 indicated that it was an excellent candidate for a *C. albicans* ortholog of *GPR1*. To study if orf19.1944 was essential for serum mediated germ tube formation it was decided to construct a strain carrying a homozygous deletion of orf19.1944.

Genetic characterisation of orf19.1944

3.2.2 Preparation of DNA for targeted disruption of orf19.1944

DNA for targeted disruption of orf19.1944 was amplified from the plasmid pBME101 (Appendix 3) using the primers orf19.1944_5'DR and orf19.1944_3'DR. (Table 3) These primers contained at their 5' end, 60 nucleotides identical to the flanking regions of orf19.1944 (Appendix 3) and at the 3' end, 20 nucleotides identical to the sequence of the *UAU1* cassette in pBME101. The resulting 4.15 kb PCR product was then used to transform *C. albicans* strain Bwp17 (Appendix 5).

3.2.3 Identification of orf19.1944 deletion strains

Arg^+ transformants were screened for the presence of the *UAU1* cassette at the orf19.1944 locus by PCR as outlined in Section 2.6.2. Appendix 4 shows the location of the PCR primers used. Of the 6 colonies screened with the primer pair orf19.1944_amp3/orf19.1944_amp5 (Appendix 4), all gave a 2.6 kb product, consistent with at least one intact copy of orf19.1944 (Figure 4; Panel A). PCR with primers orf19.1944_amp3/Arg4Det (Appendix 4) showed two colonies (#2 and #4) which gave a 1.6 kb product, consistent with the insertion of the *UAU1* cassette at the 3'end of the second copy of orf19.1944 (Figure 4; Panel B). For colonies #2 and #4 a PCR with primer pair orf19.1944_5amp2/5Arg4Det produced a 1.58 kb product, consistent with the correct insertion of the *UAU1* cassette at the 5' end of orf19.1944 (Figure 4; Panel C).

In order to further confirm that the *UAU1* cassette had integrated into the genome as intended the PCR products obtained with the orf19.1944_amp3/Arg4Det or orf19.1944_5amp2/5Arg4Det primer pairs were sequenced. Insertion of the *UAU1* cassette in the correct ORF would be confirmed by the unique sequence located between residues complementary to the flanking primers and the region incorporated into the 4.15 kb PCR product used for the disruption. In both strains, these unique identifying sequences were present as predicted. The data for one of the sequencing reactions is shown in Figure 5. The two heterozygous deletions were then renamed Bgt1a and Bgt1b (**Bwp17 G**lucose **T**ransporter **1**), respectively.

3.2.4 Identification of homozygous orf19.1944 deletions

Arg^+/Ura^+ colonies were obtained using the heterozygous deletion strain Bgt1b on YNB plates (with sucrose or mannitol substituted for glucose, Section 2.2.1). Large numbers of colonies were obtained during this selection, and twenty from each carbon source were selected at random to be screened by PCR. The orf19.1944_amp3/orf19.1944_amp5 primer pair was used to detect the absence of orf19.1944 (no 2.6 kb product) and the presence of a recombined Ura3 segment of the *UAU1* cassette (expected product: 1.8 kb). Detection of the 3' end of the *UAU1* cassette was carried out using the primer pair orf19.1944_amp3/Arg4Det (Appendix 4, expected size: 1.6 kb).

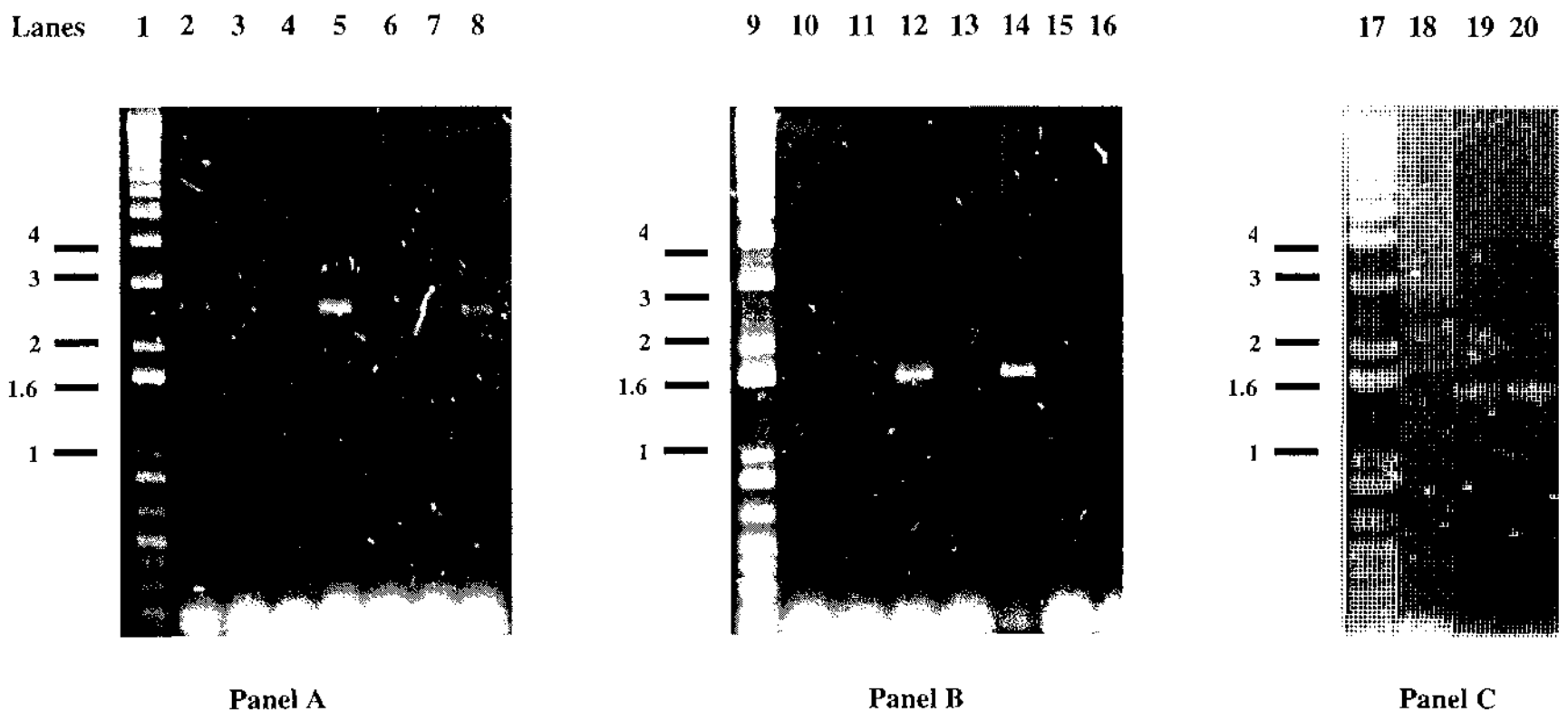


Figure 4 Screen for heterozygous deletions of orf19.1944

PCR was performed with primers orf19.1944_amp3 and orf19.1944_amp5 (**Panel A**), primers orf19.1944_amp3 and Arg4Det (**Panel B**) or primers orf19.1944_5amp2 and 5Arg4Det (**Panel C**) and genomic DNA from the parental strain Bwp17 (**lanes 2, 10 and 18**), colonies 1 – 6 (**lanes 3 – 8 and 11 – 16 respectively**) or colonies 2 and 4 (**lanes 19 and 20 respectively**). Lanes 1, 9 and 17 contained the 1Kb^{+} ladder as size standards.

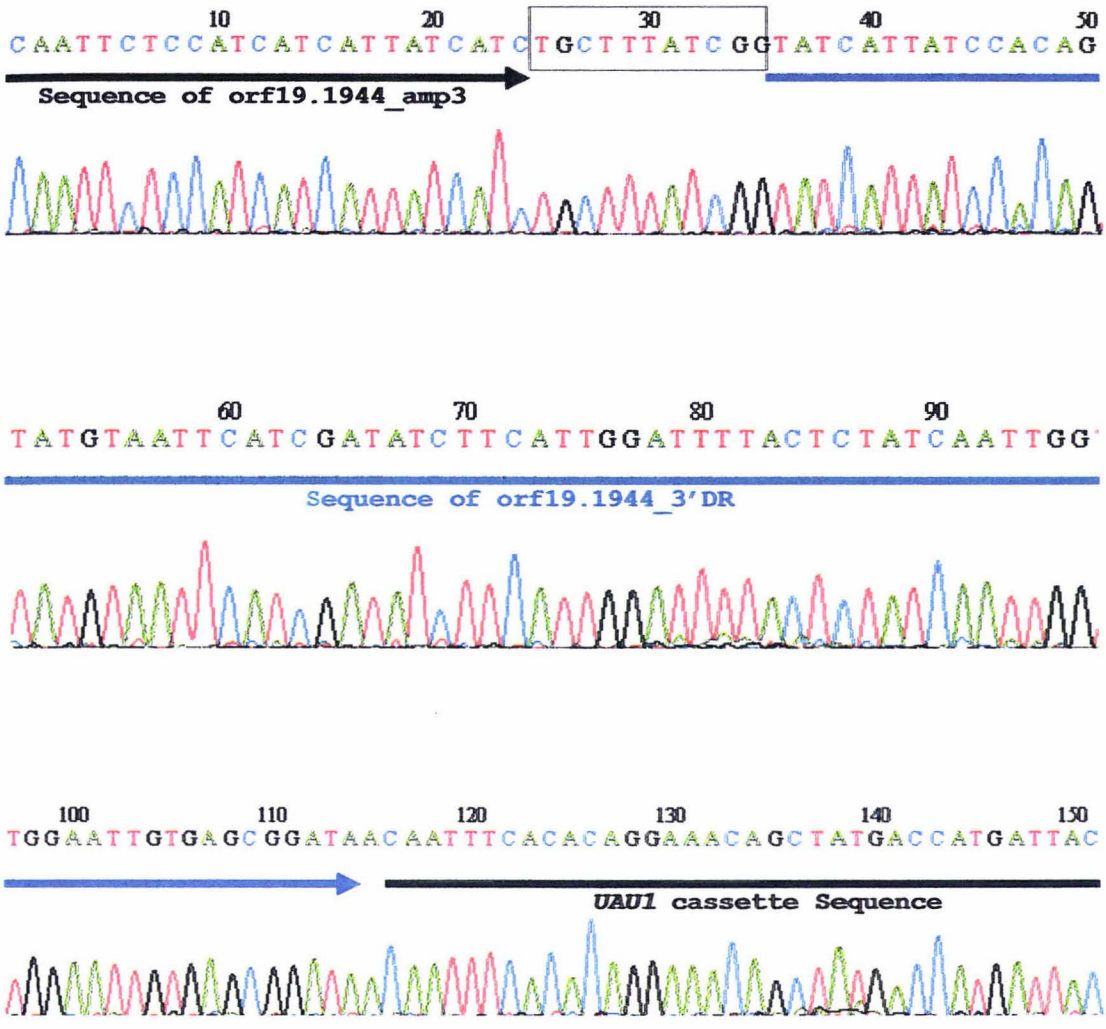


Figure 5 Sequence of the region flanking the 3'-end of the deleted orf19.1944 in the heterozygous deletion mutant Bgt1b

The PCR product obtained with primers orf19.1944_amp3 and Arg4Det was sequenced with primer *UAU1*_ComSeq. The chromatogram shows the sequence identical to the primer binding sites and the unique intervening sequence (**Boxed**) identifying the targeted ORF.

PCR of 20 sucrose grown colonies identified eighteen of them as strains carrying a third copy of orf19.1944, and two carrying a homozygous deletion of orf19.1944 (Results not shown). PCR of 20 mannitol grown colonies identified three of them as strains carrying a third copy of orf 19.1944, and seventeen carrying a homozygous deletion of orf19.1944 (Figure 6 and results not shown).

A second PCR on four mannitol grown strains, two carrying a third copy of orf19.1944 (renamed Tgt1a and Tgt1b) and two carrying a homozygous deletion of orf19.1944 (renamed Dgt1c and Dgt1d), the parental strain Bwp17 and heterozygous deletion strain Bgt1b, was done to confirm the genotype of these mutants. Another primer pair was included in this PCR. The orf19.1944_amp/1944INTPRIMER primer pair was used to confirm the absence of orf19.1944 (no 0.56 kb product). As expected the homozygous deletion strains (Dgt1c, Dgt1d) produced a 1.8 kb Ura3 band (Figure 6; Panel A), and no products from orf19.1944 itself (Figure 6; Panel A and B), and confirming the absence of orf19.1944. They also produced bands of the expected size, consistent with the correct insertion of the *UAU1* cassette at both ends of orf19.1944 (Figure 6, Panel C and D). Strains carrying a third copy of orf19.1944 (Tgt1a, Tgt1b) produced both a 2.6 kb and a 0.58 kb product from orf19.1944, as well as the 1.8 kb Ura3 band (Figure 6; Panel A and B). These colonies also produced products consistent with the correct insertion of the *UAU1* cassette into both ends of orf19.1944 (Figure 6; Panel C and Panel D). The controls produced the expected results (Figure 6). Six attempts were made to generate Arg^+/Ura^+ colonies from Bgt1a, but none were obtained. The reasons for this are not clear. Perhaps this strain carries some additional undetected mutation either within the *UAU1* cassette itself or elsewhere in the genome.

3.2.5 Phenotypic characterisation of the orf19.1944 homozygous deletion strains Dgt1a and Dgt1b

No growth difference between the homozygous deletion strains Dgt1c, Dgt1d and the parental strain Bwp17 was observed on: hexoses (mannose, galactose, fructose, and glucose), the hexol mannitol, disaccharides (sucrose and maltose) or the trisaccharide raffinose. Germ tube formation by both Dgt1c and Dgt1d was assayed using both liquid and agar based germ tube assays and compared to the parental strain Bwp17. The heterozygote Bgt1b and triplication derivatives Tgt1a and Tgt1b were included as

controls. In serum, serum filtrate and glucose there was no statistically significant difference ($P > 0.05$; Students *t*-test) between either homozygous deletion strain and the parental strain Bwp17 (Table 11). Both homozygous deletion strains formed germ tubes in buffered glucose and dialysed serum. However for one of them (Dgt1c) there was a statistically significant difference ($P < 0.05$; Students *t*-test) when compared to Bwp17. Both homozygous deletion strains were derived from the same culture of Bgt1b, and the difference observed between them cannot be explained without further investigation using additional mutants carrying a homozygous deletion of orf19.1944. No effect on filamentation was observed under nitrogen limiting conditions. The orf19.1944 homozygous deletion strains Dgt1a and Dgt1b were both able to form filaments on SLAD and spider agar media. In the embedded agar assay both Dgt1c and Dgt1d were unable to undergo filamentation, whereas both heterozygous deletion strains, the triplication derivative strains, and parental strain Bwp17 produced filaments (Figure 7). Both homozygous deletion mutants failed to produce filaments even after a further seven days incubation.

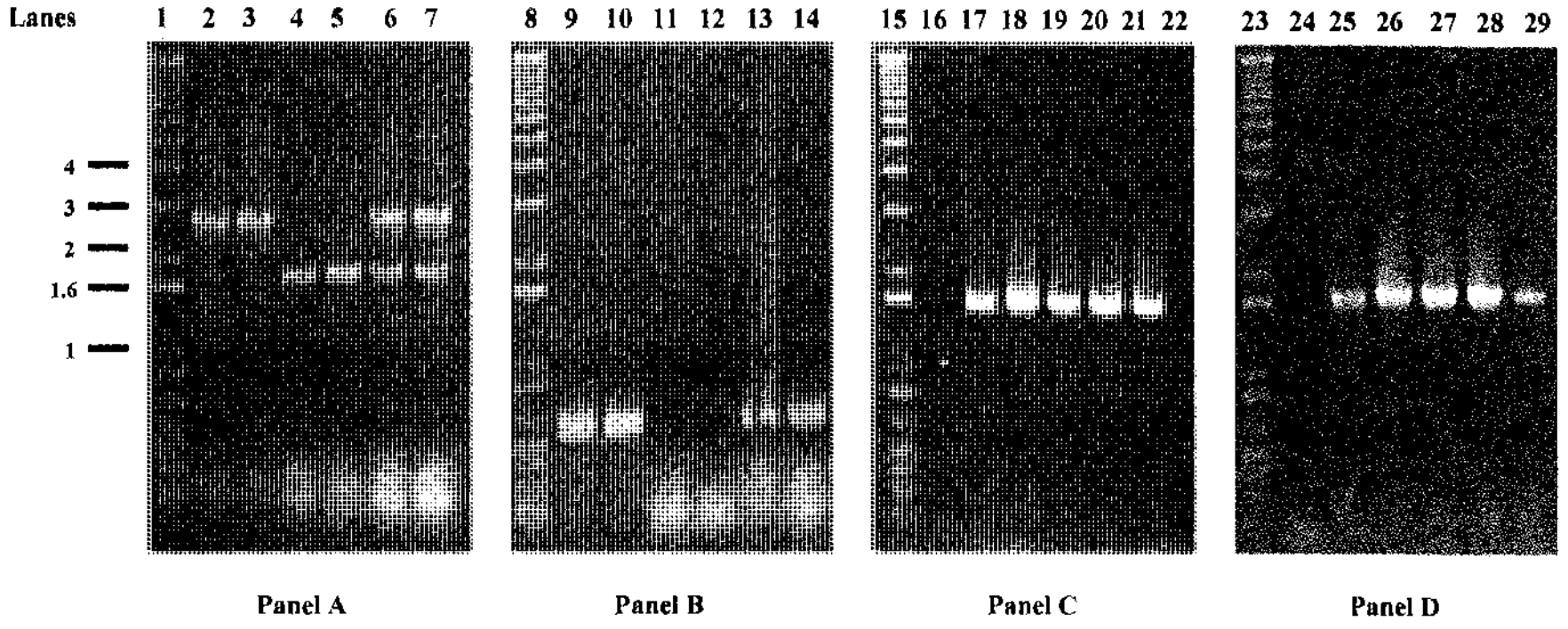


Figure 6 PCR detection of homozygous deletion strains Dgt1c and Dgt1d

PCR was performed with primers orf19.1944_amp3 and orf19.1944_amp5 (**Panel A**), primers orf19.1944_amp3 and orf19.1944INTPRIMER (**Panel B**), primers orf19.1944_amp3 and Arg4Det (**Panel C**) or primers orf19.1944_5amp2 and 5Arg4Det (**Panel D**) and genomic DNA from the parental strain Bwp17 (**lanes 2, 9, 16 and 24**) or strains Bgt1b (**Lanes 3, 10, 17, 25**), Dgt1c (**Lanes 4, 11, 18, 26**), Dgt1d (**Lanes 5, 12, 19, 27**), Tgt1a (**Lanes 6, 13, 20, 28**) and Tgt1b (**Lanes 7, 14, 21, 29**), respectively. Lanes 1, 8, 15 and 23 contained the 1Kb$^+$ ladder as size standards.

Strains	Germ Tube Formation (%) in					
	Serum	Glucose	Buffered glucose	Dialysed Serum	Dialysed Serum with glucose	Serum flitrate
Bwp17	97 ± 5 (6)	5 ± 10 (6)	32 ± 23 (6)	50 ± 42 (6)	94 ± 8 (6)	97 ± 4 (6)
Bgt1b	100 ± 0 (6)	28 ± 32 (6)	63 ± 24 (6)	73 ± 24 (6)	100 ± 1 (6)	93 ± 18 (6)
Tgt1a	94 ± 9 (6)	17 ± 17 (6)	47 ± 33 (6)	69 ± 21 (6)	93 ± 8 (6)	87 ± 30 (6)
Tgt1b	93 ± 11 (6)	5 ± 8 (6)	21 ± 20 (6)	62 ± 34 (6)	92 ± 11 (6)	78 ± 32 (6)
Dgt1c	100 ± 1 (6)	37 ± 36 (6)	65 ± 23 (6)#	98 ± 3 (6)#	100 ± 1 (6)	98 ± 3 (6)
Dgt1d	100 ± 0 (6)	14 ± 18 (6)	35 ± 19 (6)	84 ± 23 (6)	96 ± 8 (6)	90 ± 16 (6)

Table 11 Germ tube formation of orf19.1944 homozygous deletion strains

Cells were assayed for germ tube formation as outlined in Section 2.4.1, in serum (10% v/v), glucose (0.5mM), buffered glucose (50mM BICINE pH 8.0 with 0.5mM glucose), dialysed serum (equivalent to 10% v/v serum), dialysed serum with glucose serum (equivalent to 10% v/v serum with 0.5mM glucose), and serum filtrate (equivalent to 10% v/v serum), and results presented as the average ± standard deviation for the number of experiments shown in parenthesis. No germ tube formation was observed in water and buffer only controls.

Statistically significantly different compared to the parental strain Bwp17, assayed under the same conditions (student's t-test; $P < 0.05$).

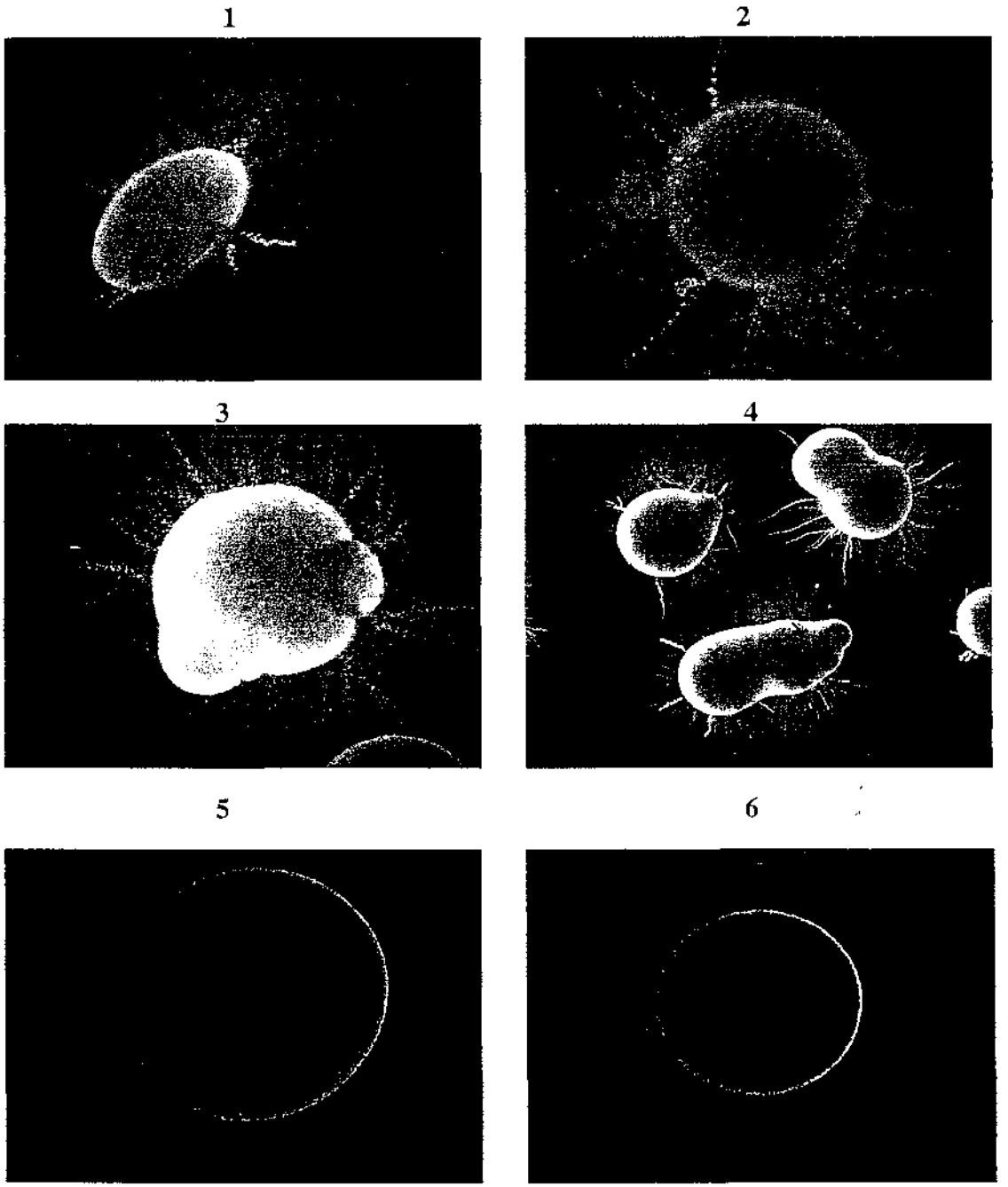


Figure 7 Germ tube formation of orf19.1944 homozygous deletion strains on embedded agar

Cells were assayed under embedded conditions as outlined in Section 2.4.2. Photos are **1**, Bwp17 (Parental strain); **2**, Bgt1b (Heterozygous deletion strain); **3**, Tgt1a (Triplication derivative strain); **4**, Tgt1b (Triplication derivative strain); **5**, Dgt1c (Homozygous deletion strain); and **6**, Dgt1d (Homozygous deletion strain).

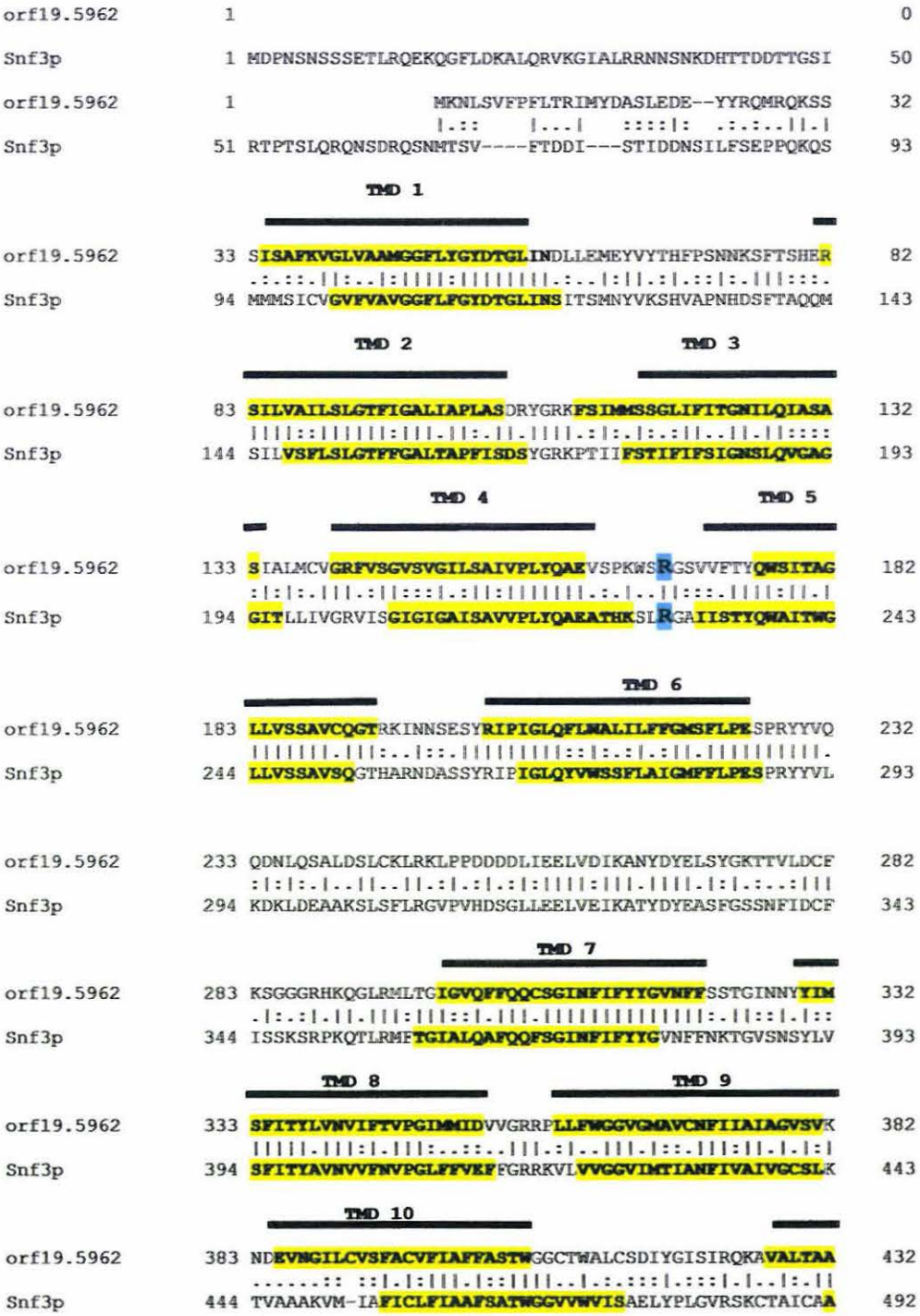
3.3 Characterisation of orf19.5962

Orf19.5962 was chosen as a candidate receptor encoding gene based on its homology to the high affinity glucose receptor *SNF3* from *Saccharomyces cerevisiae*. The putative gene product was characterised *in silico* (Section 2.8) and a strain carrying a homozygous deletion of orf19.5962 was created and its ability to form germ tubes determined (Section 2.4.1).

3.3.1 *in silico* characterisation of orf19.5962

A 748 residue protein was predicted to be encoded by orf19.5962 (Section 2.8.1). The BLASTP program (Section 2.8.1) was used to assign a putative function to this gene product by comparing the amino acid sequence to a database of fungal genome DNA sequences. The BLAST search returned 198 hits with E-values under 1e-10 all of which had roles in sugar sensing or transport. Ten hits were found with E-values under 1e-100 (Appendix 2). The two highest were to *SNF3* from *S. cerevisiae*, whilst the next highest was to *RGT2* from *S. cerevisiae*. The fifth highest hit was to orf19.7094 from *C. albicans*, which was annotated as a *SNF3* ortholog, but the length of its C-terminal tail was considered to be insufficient to act in a signalling capacity, and this ORF is considered more likely to encode a sugar transporter than a sugar receptor. The other top scoring hits were the putative *Kluyveromyces lactis* sugar sensor *RAG4*, and the monosaccharide transporters *AmMst-1* and *MSTA* from *Amanita muscaria* and *Emericella nidulans*. All had experimental evidence to support their annotation. The remaining three hits were theoretical proteins that had been assigned a putative glucose sensing or transport function through sequence similarity. The BLASTN program (Section 2.8.1) was also utilised to compare orf19.5962 and *SNF3* against the *C. albicans* genome to identify if any other ORFs with high homology were present. For orf19.5962 three ORFs were identified (Results not shown), orf19.5962 itself, a putative Hexose transporter (*HXT4*), and a putative inositol transporter (*ITR1*). For *SNF3* two putative ORFs were identified with E-values higher than 1e-10 (Results not shown). One was orf19.5962; the other was orf19.7087, which was annotated as a putative *RGT2* ortholog.

A pairwise global sequence alignment (Section 2.8.1) using EMBOSS needle was used to determine that the predicted protein from orf19.5962 has 33.2% identity and similarity of 55.2% to the protein sequence of Snf3p from *S. cerevisiae* (Figure 8). These values would have been higher except that Snf3p is 144aa longer than the putative protein encoded by orf19.5962 (Figure 8).



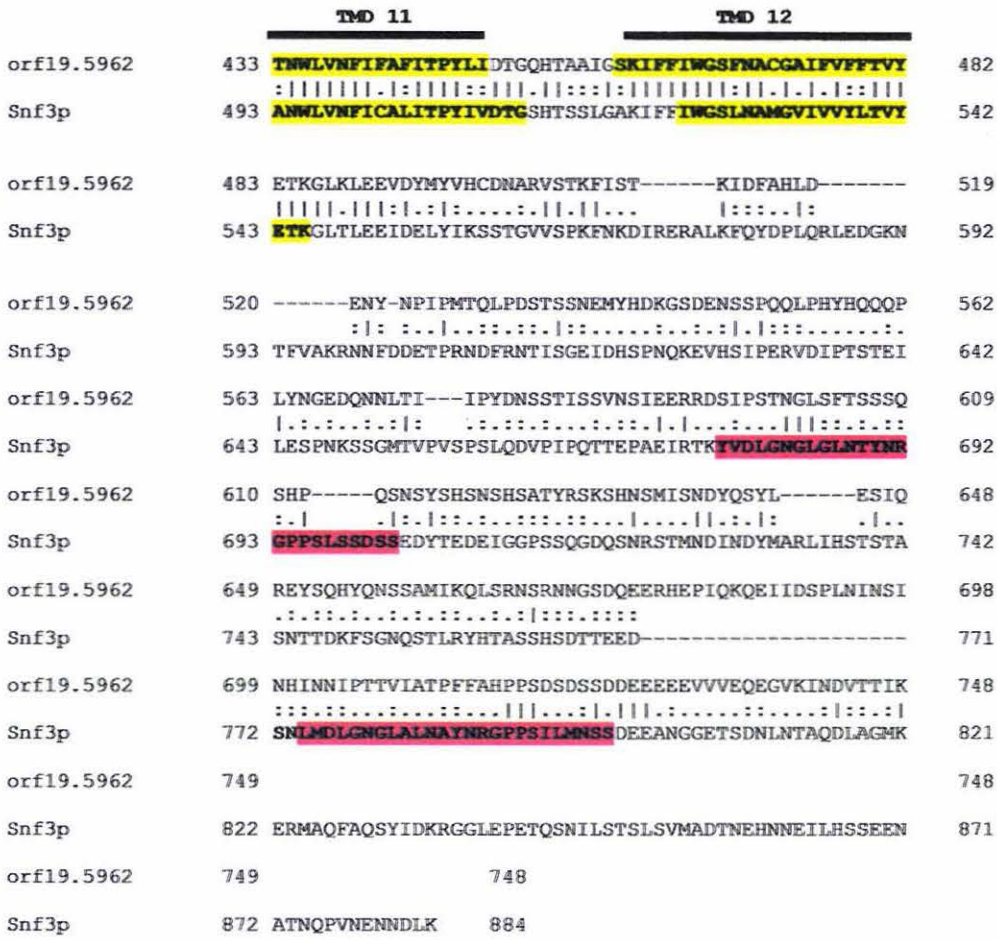


Figure 8 Global alignment of the predicted protein product of orf19.5962 against Snf3p

Snf3p is a low glucose sensor protein found in *Saccharomyces cerevisiae*. Highlighted in yellow are the predicted transmembrane domains (TMD) from orf19.5962 and Snf3p. Highlighted in teal is the conserved arginine found in all known sugar transporters, and highlighted in red are conserved repeats found to be important for the glucose signalling function of Snf3p and Rgt2p in *S. cerevisiae*.

Both rpsBLAST and SOSUI were used to search for conserved motifs in the predicted orf19.5962 protein sequence as outlined in Section 2.8.2. A conserved domain search using rpsBLAST revealed three matches. The hit scoring with an Expect (E) value lower than 1e-10 was to the sugar transporter domains found in all known sugar transporter proteins. This is consistent with the hypothesis that orf19.5962 has a role in glucose sensing. SOSUI identified twelve putative transmembrane domains (Figure 8). This is consistent with the structure of all known yeast transporters, and the

glucose sensors Rgt2p and Snf3p, which all have twelve transmembrane domains (Ozcan *et al*, 1996). The protein encoded by orf19.5962 was also shown to contain an arginine at position 168 that is conserved among all known sugar transporters in yeast, mammals, and other organisms, and the glucose sensors Rgt2p and Snf3p in *S. cerevisiae* (Ozcan *et al*, 1996). Mutation of the arginine residue to a lysine residue in the glucose receptors Rgt2p and Snf3p converts both into a dominant, constutively signalling conformation that activates *HXT* expression in the absence of glucose. These features of the protein encoded by orf19.5962 indicated that it was a good candidate for a *C. albicans* ortholog of *SNF3*. To study if orf19.5962 was involved in serum mediated germ tube formation it was decided to construct a homozygous deletion of orf19.5962.

Genetic characterisation of orf19.5962

3.3.2 Preparation of DNA for targeted disruption of orf19.5962

DNA for targeted disruption of orf19.5962 was amplified from the plasmid pBME101 (Appendix 3) using the primers orf5962_5'DR and orf5962_3'DR. (Table 5) These primers contained at their 5' end, 60 nucleotides identical to the flanking regions of orf19.5962 (Appendix 3) and at the 3' end, 20 nucleotides identical to the sequence of the *UAU1* cassette in pBME101. The resulting 4.15 kb PCR product was then used to transform *C. albicans* strain Bwp17 (Appendix 5).

3.3.3 Identification of orf19.5962 deletion strains

Arg^+ transformants were screened for the presence of the *UAU1* cassette at the orf19.5962 locus by PCR as outlined in Section 2.6.2. PCR was performed on 65 colonies, with the orf5962_amp3/orf5962_amp5 primer pair, to detect the presence of an intact copy of orf19.5962 (expected product size: 2.7 kb), and the orf19.5962_amp3/Arg4Det and the orf19.5962_amp5/5Arg4Det primer pairs to detect the correct insertion of the *UAU1* cassette into the 5' and 3' ends of orf19.5962 (Appendix 4, expected products: 1.6 kb and 1.48 kb respectively). PCR identified just three colonies (#3, #64 and #65) carrying a heterozygous deletion of orf19.5962 (Results not shown).

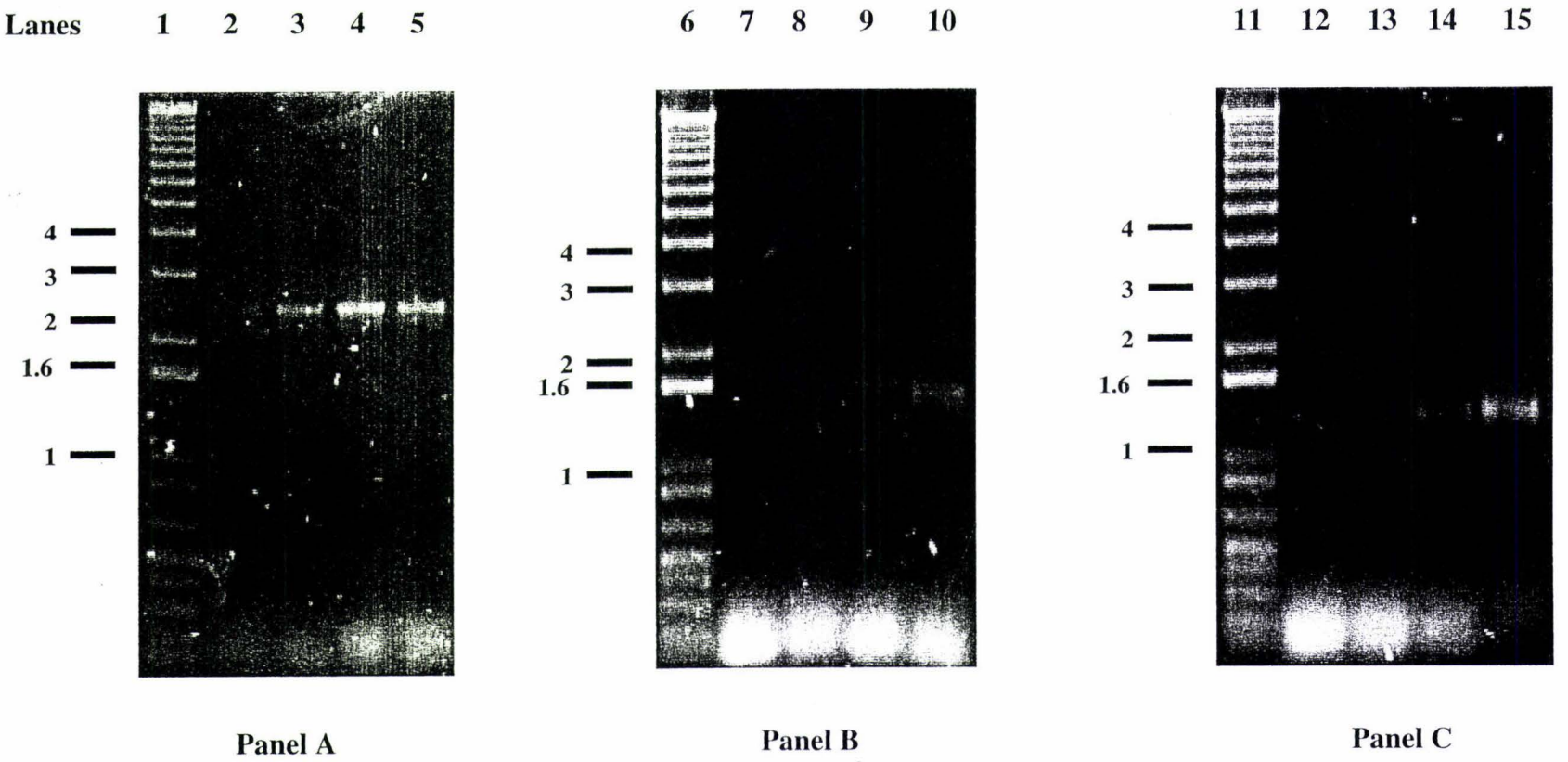


Figure 9 Screen for heterozygous deletions of orf19.5962

PCR was performed with primers orf5962_amp3 and orf5962_amp5 (**Panel A**), primers orf5962_amp3 and Arg4Det (**Panel B**) or primers orf5962_amp5 and 5Arg4Det (**Panel C**) and genomic DNA from the parental strain Bwp17 (**lanes 2, 7 and 12**) or colonies #3 (**lanes 3, 8 and 13**), #64 (**lanes 4, 9 and 14**), and #65 (**lanes 5, 10 and 15**). Lanes 1, 6 and 11 contained the $1Kb^{+}$ ladder as size standards.

A second PCR of the three heterozygous deletion strains and the parental strain Bwp17 was done to confirm their genotype. Colonies 3, 64 and 65 were confirmed as carrying a heterozygous deletion of orf19.5962, producing a 2.7 kb product consistent with an intact copy of orf19.5962 (Figure 9; Panel A), a 1.6 kb product, consistent with the correct insertion of the *UAU1* cassette at the 3'end of the second copy of orf19.5962 (Figure 9; Panel B) and a 1.48 kb product consistent with the correct insertion of the *UAU1* cassette at the 5'end of the second copy of orf19.5962 (Figure 9; Panel C).

To further confirm that the *UAU1* cassette had integrated into the genome as intended the PCR products obtained with the orf5962_amp3/Arg4Det or orf5962_amp5/5Arg4Det primer pairs were sequenced. Insertion of the *UAU1* cassette in the correct ORF would be confirmed by the unique sequence located between residues complementary to the flanking primers and the region incorporated into the 4.15 kb PCR product used for the disruption. In all three strains these unique identifying sequences were present as predicted. The data for one of the sequencing reactions is shown in Figure 10. The three heterozygotes colonies #3, #64 and #65 were then renamed Bwa1a, Bwa1b and Bwa1c, respectively.

3.3.4 Identification of homozygous orf19.5962 deletions

Heterozygous deletion strain Bwa1b was used to obtain Arg^+/Ura^+ colonies on YNB plates (with mannitol substituted for glucose, Section 2.2.1). Large numbers of colonies were obtained, and twelve were randomly selected for screening by PCR. Primer pairs used were orf5962_amp3/orf5962_amp5, to screen for the absence of orf19.5962 (no 2.7 kb product), and the presence of a recombined Ura3 segment of the *UAU1* cassette (expected product: 1.7 kb), whilst correct insertion of the *UAU1* cassette at the 3' end of orf19.5962 was confirmed using the primer pair orf5962_amp3/Arg4Det expected product: 1.6 kb). Ten strains contained a third copy of orf19.5962 and two were strains with a homozygous deletion of orf19.5962 (Results not shown).

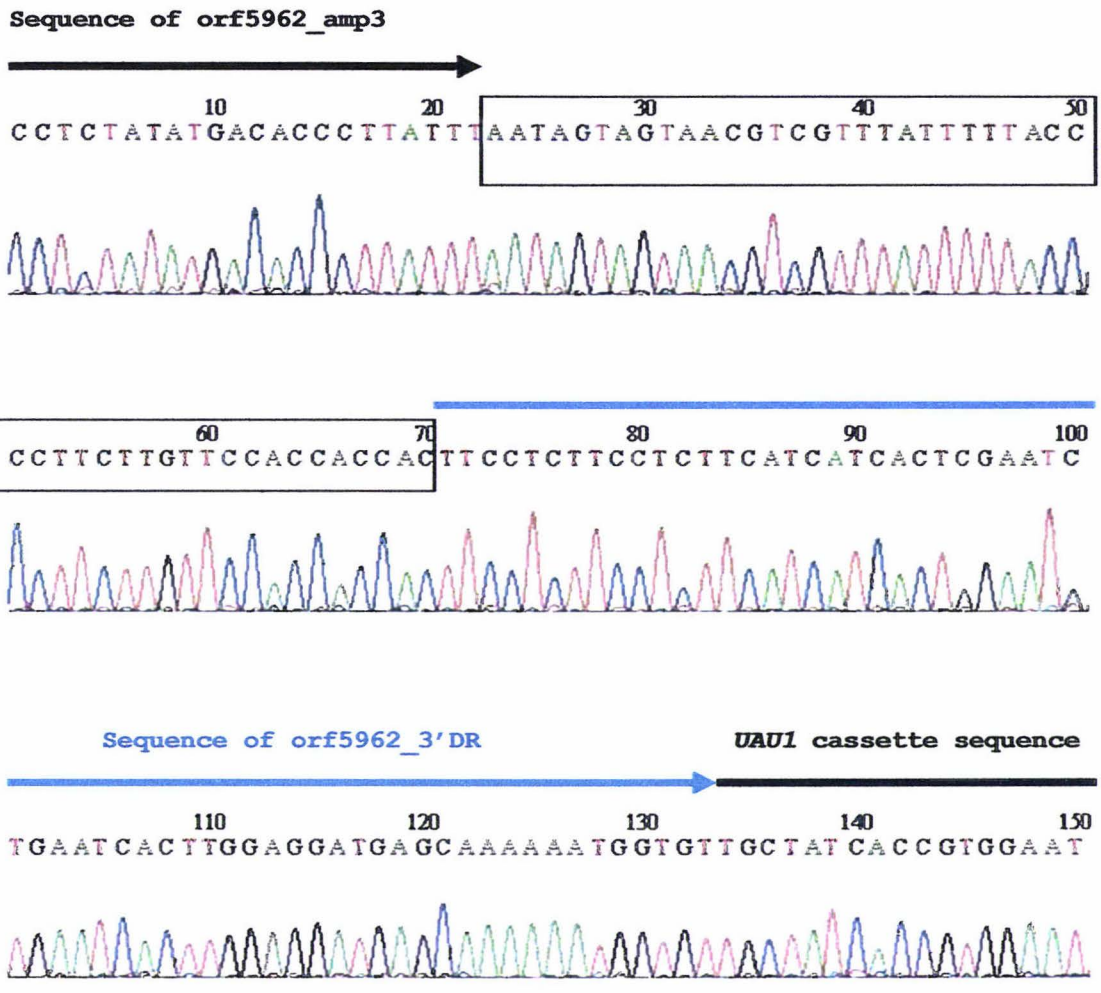


Figure 10 Sequence of the region flanking the 3'end of the deleted orf19.5962 in the heterozygous deletion mutant Bwa1b

The PCR product obtained with primers orf5962_amp3 and Arg4Det was sequenced with primer *UAU1*_ComSeq. The chromatogram shows sequence identical to the primer binding sites and the unique intervening sequence **(Boxed)** identifying the targeted ORF.

To confirm this result, a PCR of the two homozygous deletion strains (renamed Dwa1a and Dwa1b), two strains carrying a third copy of orf19.5962 (renamed Twa1a and Twa1b), the heterozygous deletion strain Bwa1b and the parental strain Bwp17 was performed. The primer pair orf5962_amp3/5962intPrime was included to confirm the absence of orf19.5962 (no 0.9 kb band). For the homozygous deletion strains the 1.7 kb Ura3 band was detected (Figure 11; Panel A), and the loss of orf19.5962, was evidenced by the absence of both the 2.7 kb and 0.9 kb bands (Figure 11; Panel A and B). In addition correct insertion of the *UAU1* cassette at both ends of orf19.5962 was confirmed (Figure 11; Panel C and D). The presence of orf19.5962 in strains carrying a third copy of orf19.5962 was confirmed (Figure 11; Panel A and B, 2.7 kb product), as was correct insertion of the *UAU1* cassette at both ends of the second copy of orf19.5962 (Figure 11; Panel C and D) and the Ura3 gene in the third copy of this ORF (Figure 11; Panel A, 1.7 kb product). Both controls produced the expected results.

Bwa1c also formed phenotypic Arg+/Ura+ colonies in numbers comparable to those achieved with Bwa1b, but these were not further characterised. Two attempts were made to generate Arg^+/Ura^+ colonies from Bwa1a, but none were obtained. The reasons for this are not clear. Perhaps this strain carries some additional undetected mutation either within the *UAU1* cassette itself or elsewhere in the genome.

3.3.5 Phenotypic characterisation of the orf19.5962 homozygous deletion strain Dwa1a

No growth difference between the homozygous deletion strain Dwa1a and the parental strain Bwp17 was observed on: hexoses (mannose, galactose, fructose, and glucose), the hexol mannitol, disaccharides (sucrose and maltose) or the trisaccharide raffinose. No difference in growth on glucose was observed at four different concentrations of glucose (0.02%, 0.2%, 2% and 20%).

The phenotype of Dwa1a was assayed using both liquid and agar based germ tube induction assays and compared to the parental strain Bwp17. The heterozygote Bwa1b and strains carrying a third copy of orf19.5962, Twa1a and Twa1b, were included as controls. Under all conditions there was no statistically significant difference (Students *t*-test; $P > 0.05$) between Dwa1a and the parental strain Bwp17 (Table 12).

In particular germ tube induction by buffered glucose or serum was not affected by the deletion of orf19.5962. Dwa1a was still able to form filaments on SLAD, spider and embedded agar media (results not shown). Thus germ tube induction by *C. albicans* under conditions if nitrogen limitation was not affected by the deletion of orf19.5962. Strain Dwa1b was obtained late in the project and its phenotype has not been characterised.

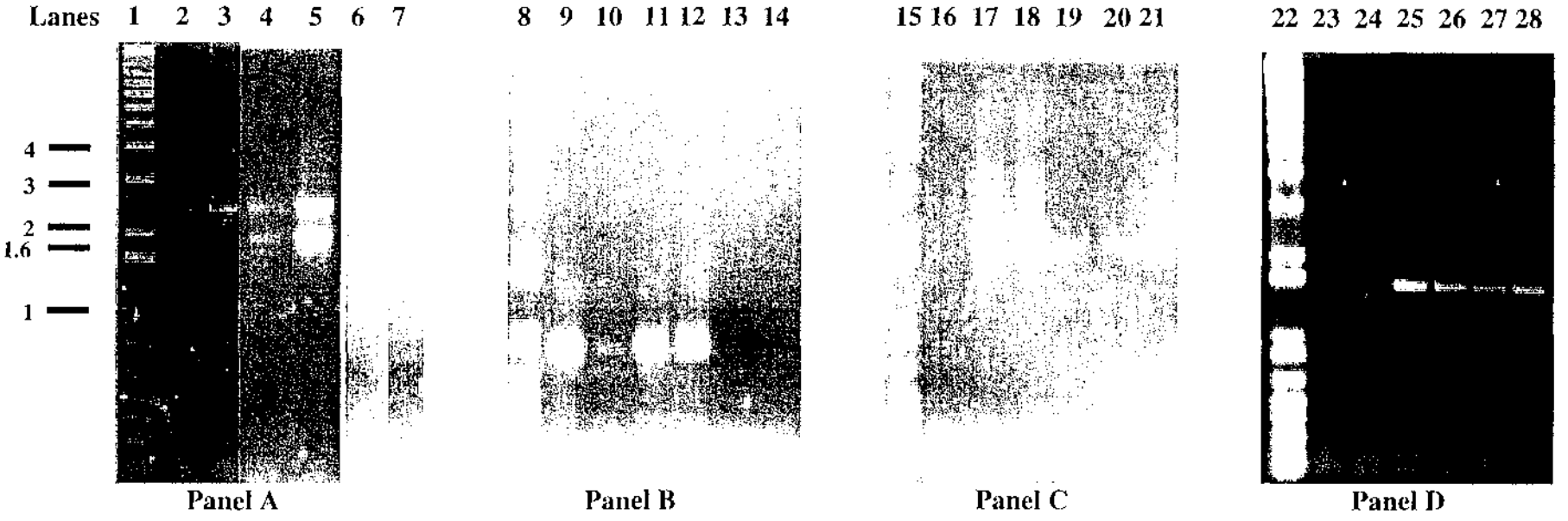


Figure 11 PCR detection homozygous deletion strains Dwa1a and Dwa1b

PCR was performed with primers orf5962_amp3 and orf5962_amp5 (**Panel A**), primers orf5962_amp3/5962intPrime (**Panel B**) primers orf5962_amp3 and Arg4Det (**Panel C**) or primers orf5962_amp5 and 5Arg4Det (**Panel D**) and genomic DNA from the parental strain Bwp17 (**lanes 2, 9, 16 and 23**), Bwa1b (**lanes 3, 10, 17 and 24**), Twa1a (**lanes 4, 11, 18 and 25**), Twa1b (**lanes 5, 12, 19 and 26**), Dwa1a (**lanes 6, 13, 20 and 27**), Dwa1b (**lanes 7, 14, 21 and 28**). Lanes 1, 8, 15 and 22 contained the 1Kb$^+$ ladder as size standards.

Strains	Germ Tube Formation (%) in					
	Serum	Glucose	Buffered glucose	Dialysed serum	Dialysed serum with glucose	Serum filtrate
Bwp17	100 ± 0 (6)	46 ± 19 (6)	92 ± 10 (6)	59 ± 29 (6)	100 ± 0 (6)	100 ± 0 (6)
Bwa1b	100 ± 0 (6)	44 ± 29 (6)	87 ± 16 (6)	60 ± 21 (6)	100 ± 0 (6)	100 ± 0 (6)
Twa1a	100 ± 0 (6)	58 ± 18 (6)	89 ± 08 (6)	79 ± 08 (6)	100 ± 0 (6)	100 ± 0 (6)
Twa1b	100 ± 0 (5)	55 ± 19 (5)	98 ± 13 (5)	79 ± 13 (5)	96 ± 6 (5)	98 ± 4 (5)
Dwa1a	100 ± 0 (6)	34 ± 32 (6)	91 ± 16 (6)	82 ± 08 (6)	100 ± 0 (6)	100 ± 0 (6)

Table 12 Germ tube formation of orf19.5962 homozygous deletion strains

Cells were assayed for germ tube formation as outlined in Section 2.4.1 in, serum (10% v/v), glucose (0.5mM), buffered glucose (50mM BICINE pH 8.0 with 0.5mM glucose), dialysed serum (equivalent to 10% v/v serum), dialysed serum with glucose serum (equivalent to 10% v/v serum with 0.5mM glucose), and serum filtrate (equivalent to 10% v/v serum) and results presented as the average ± standard deviation for the number of experiments shown in parenthesis. No germ tube formation was observed in water and buffer only controls.

No statistically significant difference (Students *t*-test; $P > 0.05$) was observed between Dwa1a and the parental strain Bwp17.

3.4 Characterisation of orf19.3668

Nantel *et al.* (2002) analysed the transcription profile of 6333 predicted ORFs in *C. albicans* and identified a small subset of ORFs, including orf19.3668, which were upregulated during the yeast to hyphal transition in serum. Orf19.3668 encodes a putative hexose transporter (*HXT*) with sequence similarity to *HXT11* from *S. cerevisiae*. *HXT11* is involved in hexose uptake and pleiotropic drug resistance. Based on this experimental evidence it was decided to investigate this ORF further, to see if it had an essential role in germ tube induction in serum or buffered glucose. The putative gene product was characterised *in silico* (Section 2.8) and a strain carrying a homozygous deletion of orf19.3668 and an undetermined amount of 5' flanking sequence upstream of this ORF, was created and its ability to form germ tubes determined (Section 2.4.1).

3.4.1 *in silico* characterisation of orf19.3668

Orf19.3668 encodes a predicted 545 residue protein. The BLASTP program (Section 2.8.1) was used to assign a putative function to this gene product by comparing the amino acid sequence to a database of fungal genome DNA sequences. The BLAST search returned over 100 hits with E-values under 1e-10, all of which were involved in either sugar transport or sensing. Seven hits were found with E-values under 1e-100 (Appendix 2). The highest was to the **H**igh Affinity **G**lucose **T**ransporter **1** (*HGT1*: orf19.4527) from *C. albicans*. The protein encoded by this gene has been functionally characterised as a high affinity glucose transporter in *C. albicans*, and was identified as orf19.4527 in the Stanford *C. albicans* genome database. The other top scoring hits were *HGT1* from *K. lactis*, *TRHXT1* from *Hypocrea jecorina* and *GTT1* from *Hypocrea lixii*, all characterised experimentally as glucose transporters, and three were theoretical proteins that had been assigned a glucose transport function through sequence similarity. Utilising BLASTN (Section 2.8.1) orf19.3668 and *HXT11* (the *S. cerevisiae HXT* with the highest homology to orf19.3668), were compared against the *C. albicans* genome to identify if any other ORFs with high homology were present. For orf19.3668 six ORFs were identified with E-values under 1e-10 (Results not shown). Orf19.3668 itself; the high affinity glucose transporter *HGT1*; orf19.9572, encoding a putative hexose transporter *HXT7*; orf19.11834, encoding an unknown protein; orf19.7094, encoding a putative *RGT2*; and orf19.3526 encoding a putative

inositol transporter *ITR2*. For *HXT11* only one putative ORF was identified with an E-value higher than 1e-10 (Results not shown). This was orf19.2021 which was annotated as a putative *HXT3* ortholog. A pairwise global sequence alignment (Section 2.8.1) using EMBOSS needle was used to determine the degree of similarity (identity of 27.9% and similarity of 51.6%) between the predicted protein from orf19.3668 and the protein sequence of *HXT11* from *S. cerevisiae* (Figure 12).

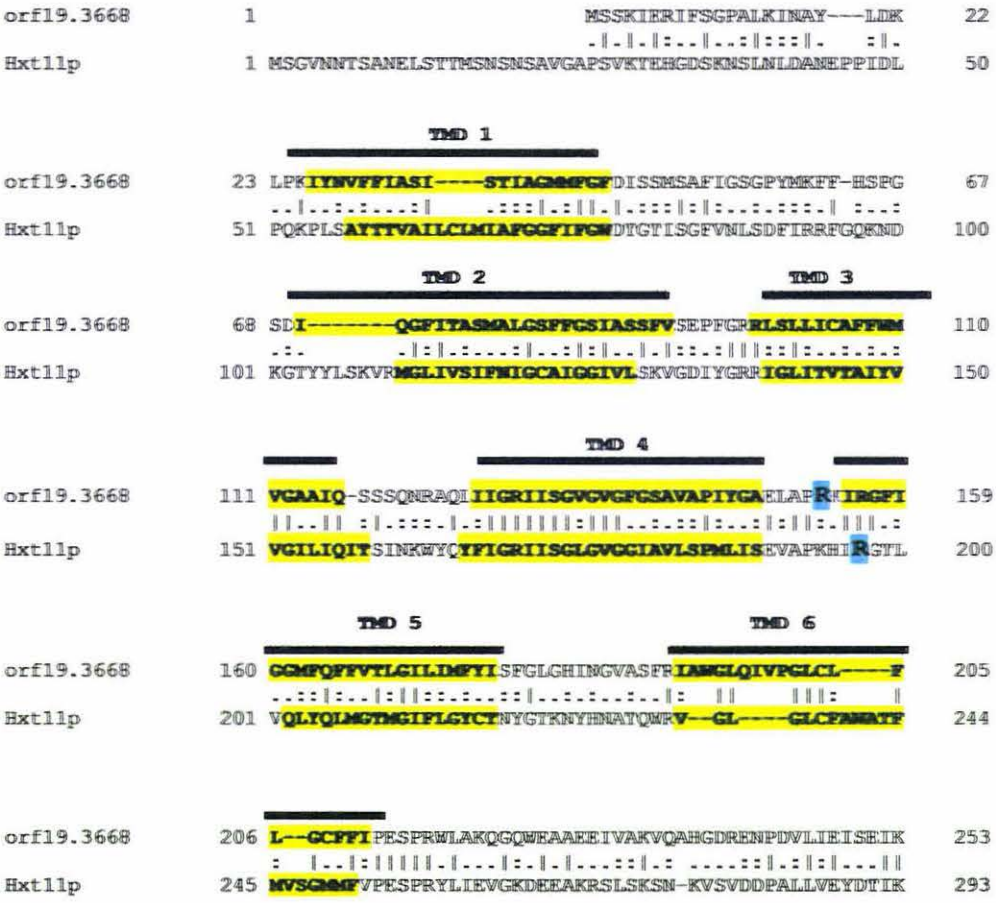


Figure 12 Global alignment of the predicted protein product from orf19.3668 and *HXT11*

HXT11 is a hexose transporter protein found in *Saccharomyces cerevisiae*. Highlighted in yellow are the predicted transmembrane domains (TMD) from orf19.3668 and *HXT11*. Highlighted in teal are known conserved amino acids found in all known hexose transporters.

Both rpsBLAST and TMHMM were used to search for conserved motifs in the predicted orf19.3668 protein sequence as outlined in Section 2.8.2. A conserved domain search using rpsBLAST revealed only one match, a sugar transporter domain that is found in all known sugar transporter proteins. This is consistent with the hypothesis of orf19.3668 having a role in glucose transport. TMHMM identified twelve putative transmembrane domains (Figure 12), a characteristic of all known fungal sugar transporters (Fan *et al.*, 2002). The putative structure of this protein indicated the presence of an arginine between transmembrane domains 4 and 5, which is conserved amongst all known 12 transmembrane domain sugar transporters (Ozcan *et al*, 1996). These features of the protein encoded by orf19.3668 indicated that it was

an excellent candidate for a glucose transporter in *C. albicans*. To study if orf19.3668 was essential for serum mediated germ tube formation it was decided to attempt construction of a strain carrying a homozygous deletion of orf19.3668.

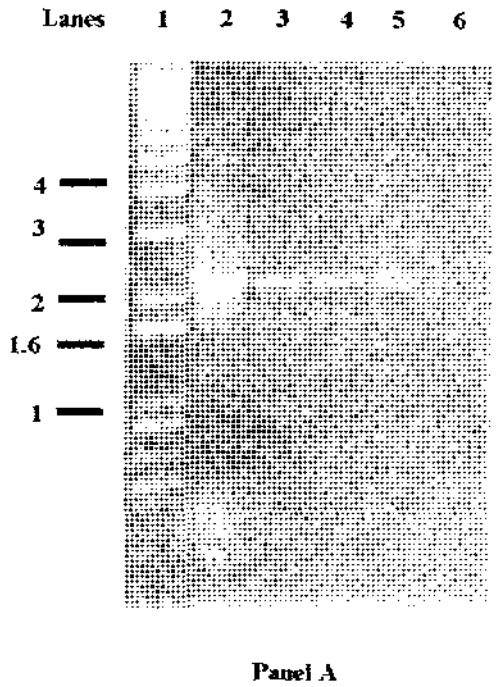
Genetic characterisation of orf19.3668

3.4.2 Preparation of DNA for targeted disruption of orf19.3668

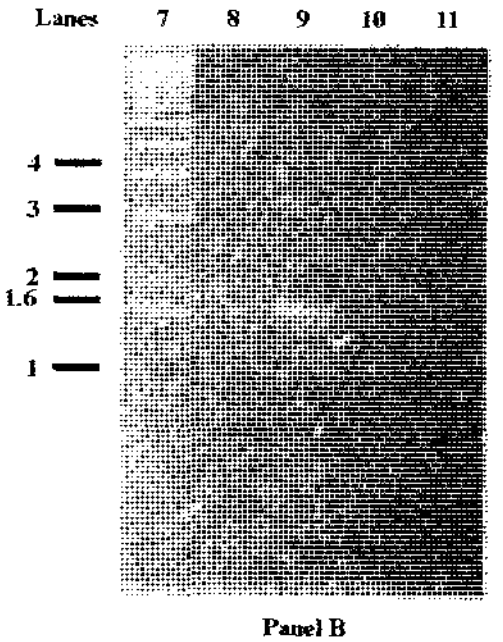
DNA for targeted disruption of orf19.3668 was amplified from the plasmid pBME101 (Appendix 3) using the primers PCF_3668__5DR and 3668_3DR (Table 4). These primers contained at their 5' end, 60 nucleotides identical to the flanking regions of orf19.3668 (Appendix 3) and at the 3' end, 20 nucleotides identical to the sequence of the *UAU1* cassette in pBME101. The resulting 4.15 kb PCR product was then used to transform *C. albicans* strain Bwp17 (Appendix 5).

3.4.3 Identification of orf19.3668 deletion strains

Arg^+ transformants were screened for the presence of the *UAU1* cassette at the orf19.3668 locus by PCR as outlined in Section 2.6.2. Appendix 4 shows the location of the PCR primers used. Fifteen colonies were screened, with primer pair orf3668_amp3/orf3668_amp5, to detect the presence of an intact copy of orf19.3668, orf3668_amp3/Arg4Det and orf3668_amp5/5Arg4Det, to show correct insertion of the *UAU1* cassette at the 3' and 5' ends of orf19.3668. One colony, colony #2, was identified as carrying a heterozygous deletion of orf19.3668, with an unknown amount of 5' flanking DNA. This was evidenced by amplification of a 2.1 kb product, consistent with an intact copy of orf19.3668 (Figure 13; Panel A), and a 1.8 kb product, consistent with the correct insertion of the *UAU1* cassette at the 3' end of the second copy of orf19.3668 (Figure 13; Panel B). No product was obtained from this, or any other clones in a screen for the correct insertion of the 5' end of the *UAU1* cassette into orf19.3668. Three separate orf19.3668_amp5 primers (Appendix 4) were used to screen a 1 kb region flanking the 5' end of orf19.3668, in an attempt to ascertain where the *UAU1* cassette had inserted in colony #2. None produced a product (not shown). The disruption primer PCF_3668__5DR was rich in A and C residues, as was the 4 kb of sequence immediately flanking the 5' end of orf19.3668. The 5' end of the *UAU1* cassette might have inserted anywhere in this region beyond the outermost orf19.3668_amp5 primer.



Panel A



Panel B

Figure 13 Screen for heterozygous deletions of orf19.3668

PCR was performed with primers orf3668_3amp and orf3668_5amp (Panel A), or primers orf3668_3amp and Arg4Det (Panel B) and genomic DNA from the parental strain Bwp17 (lane 2) or colony 1 (lanes 3, 8), 2 (lanes 4, 9), 3 (lanes 5, 10) and colony 4 (lanes 6, 11). Lanes 1 and 7 contained the 1Kb$^+$ ladder as the size standard.

In order to further confirm that the *UAU1* cassette had integrated into the 3' end of orf19.3668 correctly the PCR product obtained with the orf3668_3amp/Arg4Det primer pair was sequenced. Insertion of the *UAU1* cassette in the correct ORF would be confirmed by the unique sequence located between the residues complementary to the flanking primer and the region incorporated into the 4.15 kb PCR product used for the disruption. The orf19.3668 3' end unique identifying sequence was present as expected (Figure 14). This heterozygous Arg+ strain was renamed Bgr1a (**B**wp17 **G**lucose **R**eceptor **1a**). No other heterozygotes were obtained, and it was decided to continue with the generation of a Arg+/Ura+ strain, that would be expected to carry a deletion of orf19.3668 and an unknown amount of 5' flanking sequence. The size of the deletion would be further characterised if the phenotype of interest was obtained.

3.4.4 Identification of homozygous deletions derived from strain Bgr1a

Numerous Arg^+/Ura^+ colonies were obtained using the heterozygous deletion strain Bgr1a on YNB plates (with mannitol substituted for glucose, Section 2.2.1). Because the orf3668_5amp primer binding site was absent the six colonies were screened by PCR using the primer pair orf3668_3amp/intPRIME3668 to detect orf19.3668 (expected product: 0.82 kb) and primer pair 5'-detect/3'-detect, to detect the presence of a recombined Ura3 from the *UAU1* cassette (expected product: 1.6 kb). Detection of the 3' end of the *UAU1* cassette was carried out using primer pair orf3668_3amp/Arg4Det (expected size: 1.6 kb). All six clones were homozygous for the deletion carried by Bgr1a (Results not shown). No triplication derivatives were obtained with this mutant.

To confirm this result, the PCR was repeated for two homozygous deletion strains (Dgr1a, Dgr1b), the heterozygous deletion strain Bgr1a and parental strain Bwp17. An additional primer pair was included (Ygr189amp3/Ygr189amp5) as a control. For Dgr1a and Dgr1b the homozygosity of the deletion carried by Bgr1a, was confirmed as evidenced by the absence of a 0.82 kb orf19.3668 band (Figure 15; Panel B), the presence of 1.6 kb Ura3 band (Figure 15; Panel C), and correct insertion of the *UAU1* cassette at the 3' end of orf19.3668 (Figure 15; Panel D).

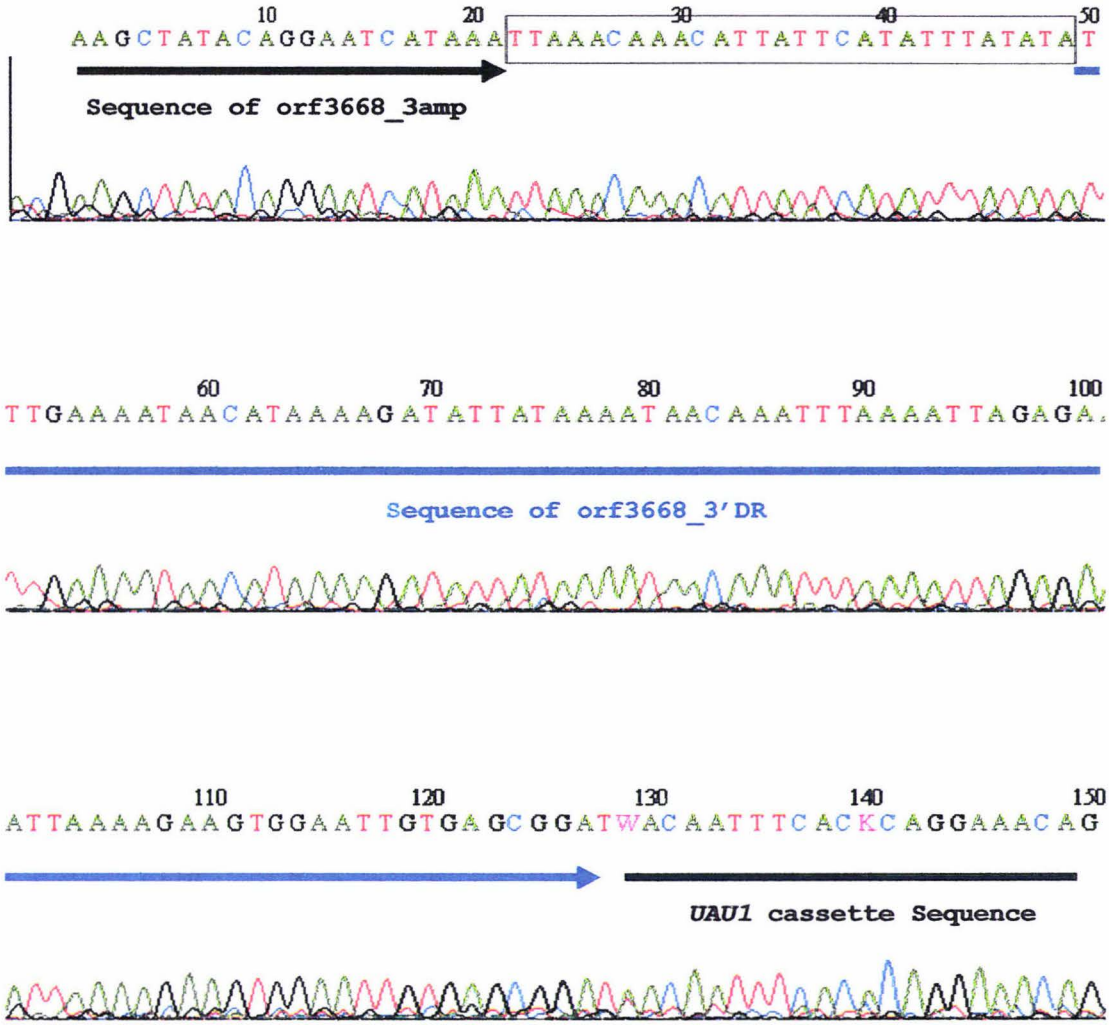


Figure 14 Sequence of the region flanking the 3' end of the deletion in the heterozygous deletion mutant Bgr1a

The PCR product obtained with primers orf3668_3amp and Arg4Det was sequenced with primer *UAU1*_ComSeq. The chromatogram shows the sequence identical to the primer binding sites and the unique intervening sequence (**Boxed**) identifying the targeted ORF.

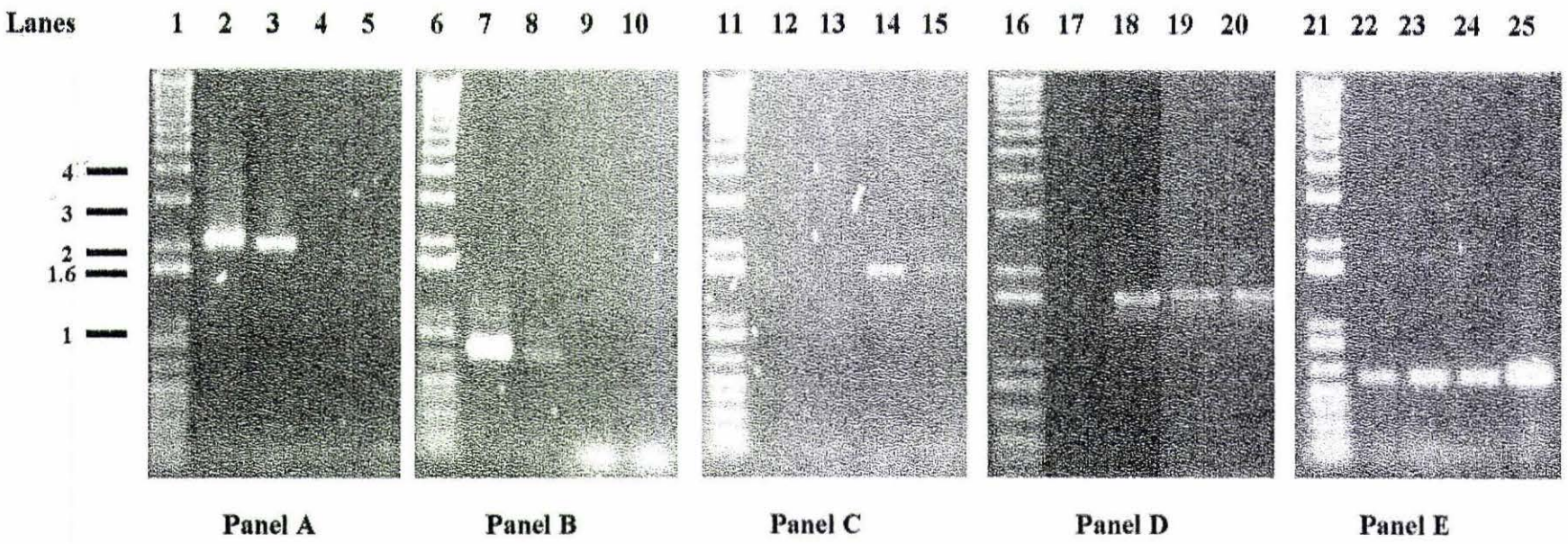


Figure 15 PCR analysis of the homozygous deletion strains Dgr1a and Dgr1b

PCR was performed with primer pair orf3668_3amp/orf3668_5amp (**Panel A**), primer pair orf3668_3amp/intPrime3668 (**Panel B**), primer pair orf3668_3amp/Arg4Det (**Panel C**), primer pair 3-detect/5-detect (**Panel D**) or primer pair Ygr189amp3/Ygr189amp5 (**Panel E**) and genomic DNA from the parental strain Bwp17 (**lanes 2, 7, 12, 17 and 22**) or strains Bgr1a (**lanes 3, 8, 13, 18, 23**), Dgr1a (**lanes 4, 9, 14, 19, 24**) and Dgr1b (**lanes 5, 10, 15, 20, and 25**). Lanes 1, 6, 11, 16 and 21 contained the $1Kb^{+}$ ladder as size standards.

C. albicans Strains	Germ Tube Formation (%) in					
	Serum	Glucose	Buffered glucose	Dialysed serum	Dialysed serum with glucose	Serum filtrate
Bwp17	100 ± 1 (6)	40 ± 22 (6)	84 ± 34 (6)	53 ± 26 (6)	99 ± 2 (6)	99 ± 2 (6)
Bgr1a	100 ± 0 (5)	46 ± 6 (5)	99 ± 3 (5)	40 ± 13 (5)	100 ± 0 (5)	100 ± 0 (5)
Dgr1a	100 ± 0 (6)	32 ± 22 (6)	91 ± 23 (6)	54 ± 26 (6)	100 ± 0 (6)	100 ± 0 (6)
Dgr1b	100 ± 0 (6)	43 ± 16 (6)	92 ± 21 (6)	53 ± 25 (6)	100 ± 0 (6)	100 ± 1 (6)

Table 13 Germ tube formation of orf19.3668 homozygous deletion strains

Cells were assayed for germ tube formation as outlined in Section 2.4.1 in, serum (10% v/v), glucose (0.5mM), buffered glucose (50mM BICINE pH 8.0 with 0.5mM glucose), dialysed serum (equivalent to 10% v/v serum), dialysed serum with glucose serum (equivalent to 10% v/v serum with 0.5mM glucose), and serum filtrate (equivalent to 10% v/v serum) and results presented as the average ± standard deviation for the number of experiments shown in parenthesis. No germ tube formation was observed in water or buffer only controls.

No statistically significant difference (Students *t*-test, $P > 0.05$) was found between Dgr1a or Dgr1b and the parental strain Bwp17.

CHAPTER FOUR: DISCUSSION

A serum filtrate (molecular mass, < 10 kDa) was found to induce germ tube levels equivalent to those induced using serum, in all the clinical isolates tested, except strain OTG10 (Section 3.2.1). Feng *et al.* (1999) also showed that a serum filtrate (molecular mass, < 1 kDa) was able to induce germ tube levels equivalent to serum in *C. albicans* strain Sc5314. Previous analysis of serum filtrate mediated germ tube formation in *C. albicans* strain A72 identified the inducer present in the serum filtrate as glucose (Hudson and Farley, unpublished).

Glucose can induce germ tube formation in all the clinical isolates of *C. albicans* tested, except strain CLB42, which was also a low responder in serum (Section 3.1.3). For ten out of the sixteen isolates tested there was no statistically significant difference between the level of germ tube induction by buffered glucose and serum. Thus, glucose is the major dialysable inducer in serum. Glucose has been previously reported to be an inducer of germ tube formation (Vidotto *et al.*, 1996, Pollack *et al.*, 1987) and may induce germ tube formation by binding to a surface receptor, which then transduces the induction signal through one or more biochemical pathways that lead to germ tube formation.

Dialysed serum (molecular mass, > 10 kDa), still retained a low level of induction potential, but was not as potent as serum or serum filtrate (Section 3.1.1). The addition of exogenous glucose alone, to the dialysed serum, was shown to increase its induction potential to levels comparable to those of serum, in the majority of the clinical isolates tested (Section 3.1.1). The active component of dialysed serum has not yet been identified.

Homozygous deletion of orf19.3668 (and an unknown region of 5' flanking sequence), orf19.1944, or orf19.5962 was unable to abrogate serum or glucose mediated germ tube formation in *C. albicans* (Section 3.2.5, 3.3.5, and 3.4.5). Thus orf19.1944, orf19.5962 and orf19.3668, do not play an essential role in serum or glucose mediated germ tube formation. No effect on filamentation was observed under nitrogen limiting conditions. The ability of orf19.1944 homozygous deletion

strains Dgt1a and Dgt1b to form filaments under nitrogen limiting conditions was surprising, since *S. cerevisiae GPR1* homozygous deletion strains have been shown to be unable to undergo pseudohyphal differentiation under nitrogen limiting conditions (Lorenz *et al*, 2000).

No growth difference between the orf19.1944, orf19.3668 and orf19.5962 homozygous deletion strains and the parental strain Bwp17 was observed on: hexoses (mannose, galactose, fructose, and glucose), the hexol mannitol, disaccharides (sucrose and maltose) or the trisaccharide raffinose. The ability of orf19.5962 homozygous deletion strain Dwa1a to grow on low concentrations of glucose was unexpected because strains carrying a homozygous deletion of the *S. cerevisiae* ortholog *SNF3* are unable to grow on low concentrations of glucose. It may be that orf19.5962 is neither a low or high affinity glucose receptor. If it is not involved in glucose sensing then it may be a glucose transporter with an unusually long C-terminal domain. In *S. cerevisiae* there are 34 known sugar permease proteins, of which 18 belong to the hexose transporter subfamily, and only a strain carrying a concurrent knockout of 20 of these transporter genes is unable to uptake hexoses (Wieczorke *et al*, 1999). If orf19.5962 is a hexose transporter then a strain carrying a homozygous deletion may show no obvious phenotype, unless genes with similar function are concurrently knocked out. Transcription profiling of 20 putative hexose transporters in *C. albicans*, in media containing 5%, 2% and 0.2% glucose (Fan *et al.*, 2002) showed that orf19.5962 is expressed in media containing 5% glucose. In contrast the *S. cerevisiae* glucose receptor *SNF3* is glucose repressible, and is only induced under low glucose conditions (Ozcan *et al*, 1996). Orf19.5962 has an expression profile that is more similar to that of *HXT 11* and *12* from *S. cerevisiae* (Ozcan *et al*, 1999). The role of these two genes in *S. cerevisiae* is, however, yet to be fully elucidated. Fan *et al.* (2002) identified another putative *SNF3* candidate, based on phylogenetic linkage and expression analysis, which they named *HGT12*. The predicted protein product doesn't have a C-terminal tail like Snf3p; however this is not an absolute requirement for a glucose receptor. Glut2p, a low-affinity glucose transporter which is required for glucose induced expression of insulin in pancreatic β-bells, does not have a large C-terminal extension (Arbuckle *et al,* 1996). Thus *HGT12* may be an excellent candidate for future studies.

C. albicans strains carrying a homozygous deletion of orf19.1944 were unable to form germ tubes in response to embedded conditions (Section 3.1.5); a phenotype shared by strains that carry a homozygous deletion of the transcription factor, Candida **Z**inc **F**inger 1 (*CZF1*), (Brown *et al.*, 1999). No other components of the *CZF1* pathway have been identified. Identifying if orf19.1944 is a component of this pathway could be a focus of future research, using epistasis studies of strains with homozygous deletions of *CZF1* and orf19.1944.

To date, the only positively acting biochemical pathway, in *C. albicans* to encode components essential for serum mediated germ tube formation is the cAMP/PKA pathway (Ernst and Bockmuhl, 2001; Rocha *et al.*, 2001; Feng *et al.*, 1999). Many components have been characterised in the cAMP/PKA pathway, but none are receptors. The only receptor identified thus far, with a link to germ tube formation in *C. albicans* is the amino acid sensor Csy1p, which has been shown to be required for filamentation on solid media that contain amino acids (Brega *et al.*, 2004). However the filamentation pathway it signals through is still to be identified.

Although the receptor that mediates serum and glucose induction of germ tube formation in *C. albicans* was not identified, a search of the Stanford Contig 19 Assembly (http://www-sequence.stanford.edu/group/candida/) has revealed at least 20 ORFs encoding putative glucose transporters/receptors. The possible role these ORFs may play in glucose and serum mediated germ tube formation could be determined by the generation of strains carrying a homozygous deletion of each ORF. Identification of the receptor could also be facilitated by isolating it from cell membrane extracts through column purification; using matrix bound glucose or glucose analogs.

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APPENDIX 1: DATA FOR PLOTTING STANDARD CURVES OF CELLS/ML vs OD_{600}

Sc5314 Log Cell Concentration

OD_{600}	10^{-7} Number of Cells per mL
0.00	0.00
0.23	0.59
0.39	1.01
0.61	1.20
0.82	2.09

Sc5314 Stationay Cell Concentration

OD_{600}	10^{-7} Number of Cells per mL
0.00	0.00
0.19	0.34
0.44	0.80
0.64	1.25
0.81	1.87

A72 Log Cell Concentration

OD_{600}	10^{-7} Number of Cells per mL
0.00	0.00
0.21	0.67
0.41	0.95
0.64	1.14
0.82	1.82

A72 Stationay Cell Concentration

OD_{600}	10^{-7} Number of Cells per mL
0.00	0.00
0.18	0.44
0.42	0.69
0.61	0.99
0.79	1.44

Duplicate cell counts were averaged and then multiplied by 10^4 to give the concentration in cells/ml at the given OD_{600}.

APPENDIX 2: ORF *IN SILICO* CHARACTERISATION

BLASTP Results for orf19.1944

Sequences producing significant alignments:	Score (bits)	E Value
gi|6320170|ref|NP_010249.1| G-protein-coupled receptor at p...	167	1e-39
gi|7493833|pir||JC5808 G protein-coupled receptor 1 - yeast...	167	1e-39
gi|32411373|ref|XP_326167.1| predicted protein [Neurospora ...	99	3e-19
gi|38104760|gb|EAA51281.1| hypothetical protein MG08803.4 [...	92	4e-17
gi|19075728|ref|NP_588228.1| glucose-triggered adenylate cy...	80	1e-13

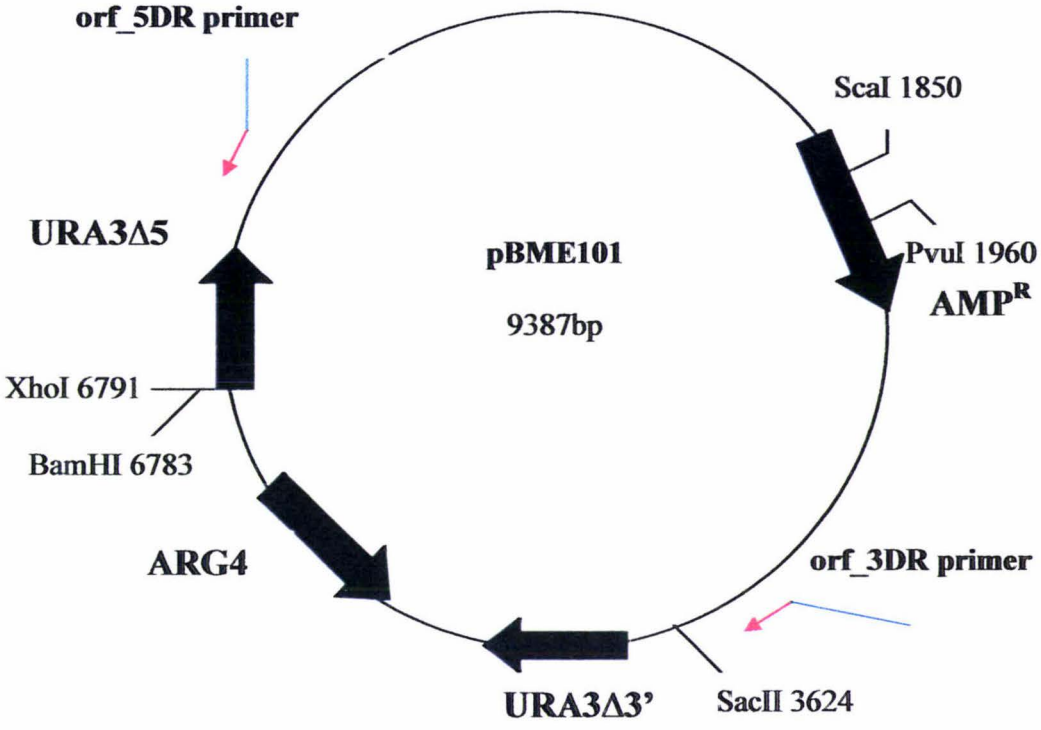
BLASTP Results for orf19.5962

Sequences producing significant alignments:	Score (bits)	E Value
gi|6320007|ref|NP_010087.1| glucose sensor; Snf3p [Saccharo...	527	e-148
gi|1351078|sp|P10870|SNF3_YEAST High-affinity glucose trans...	526	e-148
gi|6320063|ref|NP_010143.1| plasma membrane glucose sensor;...	508	e-142
gi|2440082|emb|CAA75114.1| putative glucose sensor [Kluyver...	501	e-140
gi|24461762|gb|AAN62329.1| putative glucose sensor protein ...	452	e-125
gi|32407620|ref|XP_324320.1| hypothetical protein [Neurospo...	417	e-115
gi|32416816|ref|XP_328886.1| hypothetical protein [Neurospo...	404	e-111
gi|2258125|emb|CAB06078.1| AmMst-1 [Amanita muscaria]	399	e-109
gi|38106813|gb|EAA53075.1| hypothetical protein MG06203.4 [...	390	e-107
gi|25004737|emb|CAC80843.1| MSTA protein [Emericella nidulans]	371	e-101

BLASTP Results for orf19.3668

Sequences producing significant alignments:	Score (bits)	E Value
gi|6016201|sp|O74713|HGT1_CANAL High-affinity glucose trans...	1007	0.0
gi|1346290|sp|P49374|HGT1_KLULA High-affinity glucose trans...	548	e-154
gi|40745016|gb|EAA64172.1| hypothetical protein AN2466.2 [A...	413	e-114
gi|40742285|gb|EAA61475.1| hypothetical protein AN9184.2 [A...	408	e-112
gi|32416760|ref|XP_328858.1| hypothetical protein [Neurospo...	379	e-103
gi|38505355|gb|AAR23147.1| glucose transporter; TrHXT1 [Hyp...	377	e-103
gi|20135735|emb|CAC81782.1| glucose transporter [Hypocrea l...	374	e-102

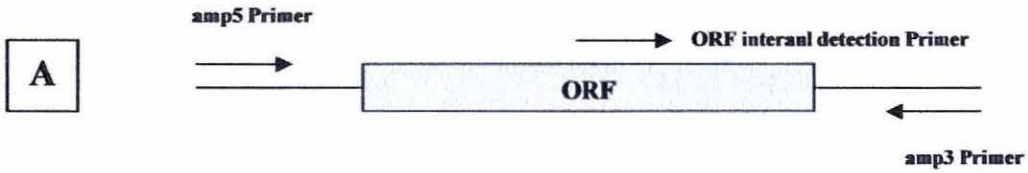
APPENDIX 3: MAP OF PLASMID pBME101



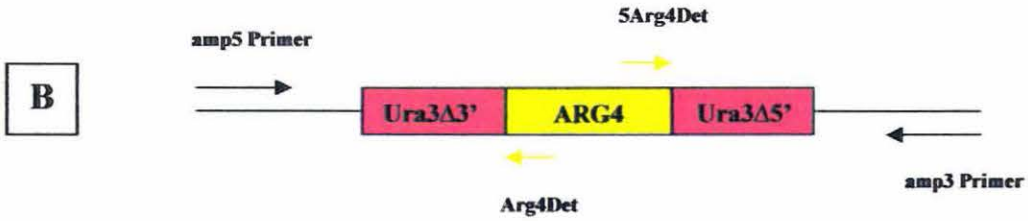
The plasmid pBME101 was used as template in PCR reactions to amplify DNA for targeted disruptions of selected ORFs. Also shown in this diagram are generalised 5DR and 3DR primers each of which contained 20 bp of sequence complementary to the pBME101 region either directly upstream or downstream of the *UAU1* cassette (highlighted in red), and 60 bp of sequence from one or other end of the target ORF (highlighted in blue).

APPENDIX 4: PCR STRATEGIES

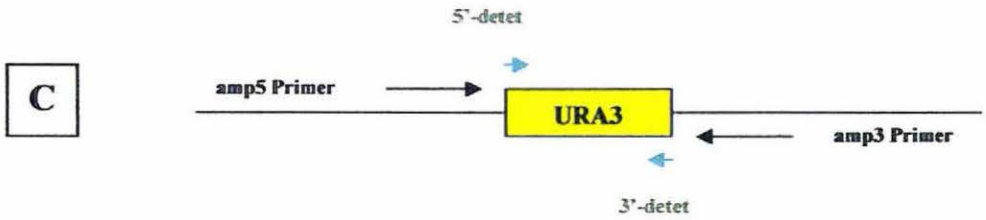
INTACT ORF



UAU1 CASSETTE INSERT

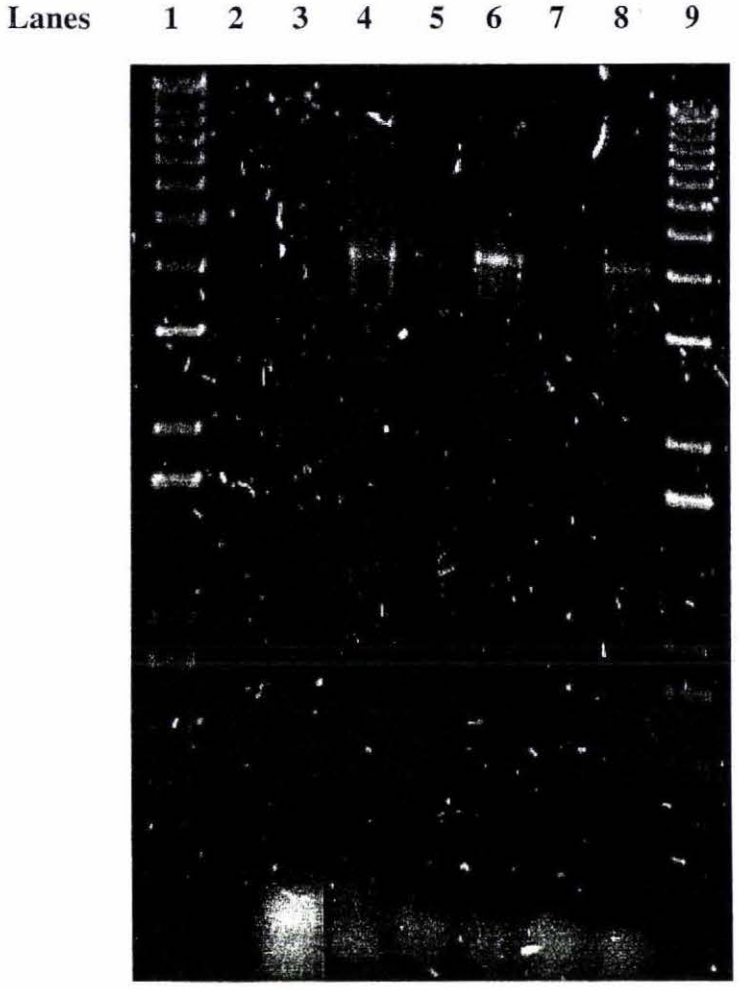


RECOMBINED URA3



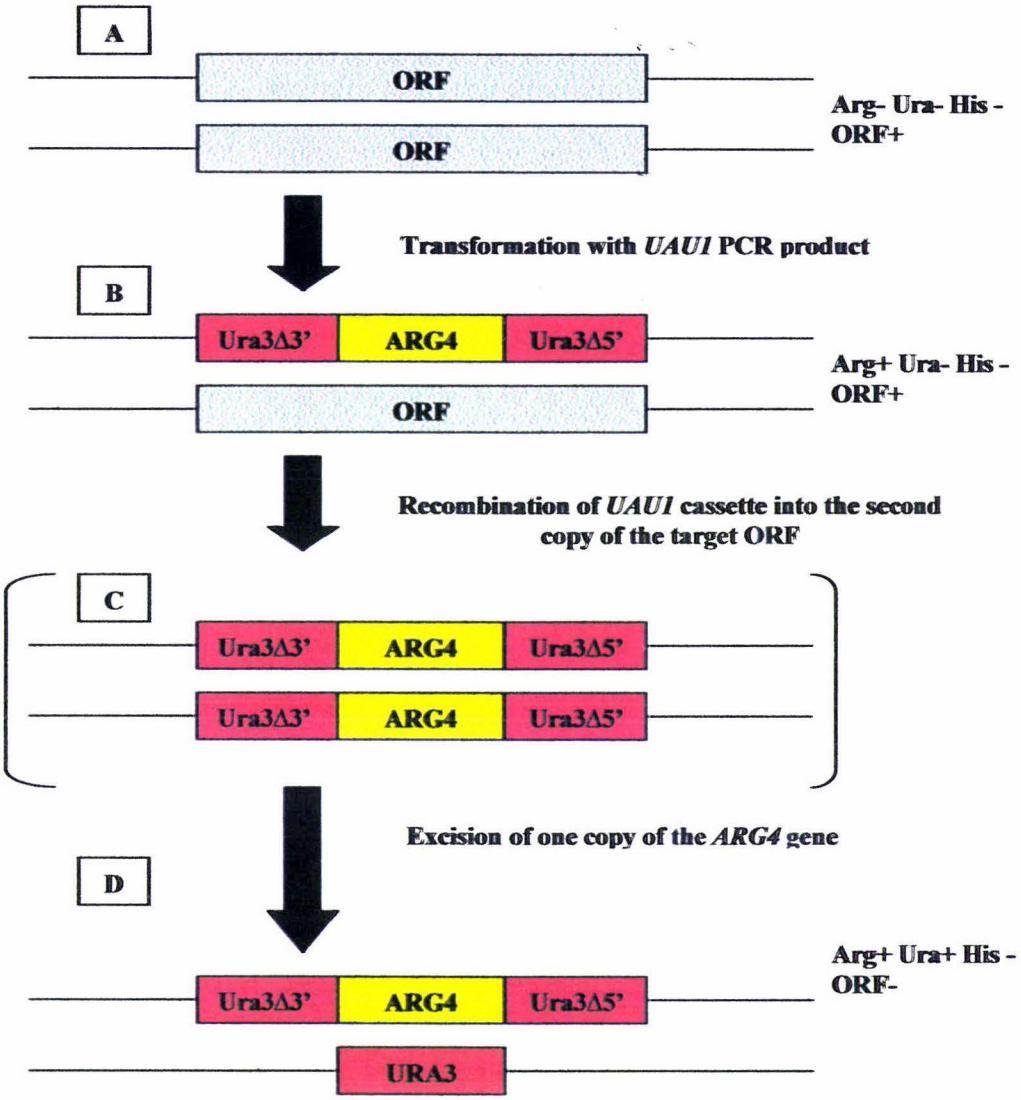
Panel A: Amplification of the intact orf for, **orf19.1944** was carried out using primer pairs orf19.1944_amp3/orf19.1944_amp5 and orf19.1944_amp3/orf19.1944INTPRIMER; **orf19.5962** using primer pairs orf5962_3amp/orf5962_5amp and orf5962_3amp/orf5962intPrime; **orf19.3668** using primer pairs orf3668_3amp/orf3668_5amp and orf3668_3amp/intPrime3668. **Panel B:** Amplification of the *UAU1* insertion in **orf19.1944** was carried out using primer pairs orf19.1944_amp3/Arg4Det and orf19.1944_amp5/5Arg4Det; **orf19.5962** using primer pairs orf5962_3amp/Arg4Det and orf5962_5amp/5Arg4Det; **orf19.3668** using primer pairs orf3668_3amp/Arg4Det and orf3668_5amp/5Arg4Det. **Panel C:** Amplification of the recombined URA3 in **orf19.1944** was carried out using primer pair orf19.1944_amp3/orf19.1944_amp5; **orf19.5962** using primer pair orf5962_3amp/orf5962_5amp; **orf19.3668** using primer pair 5'-detect/3'-detect.

APPENDIX 5: DNA FOR TARGETED DISRUPTION OF ORFs



PCR was performed with the primer pairs orf19.1944_5DR/orf19.1944_3DR (**lanes 3 and 4**), orf19.3668_5DR/orf19.3668_3DR (**lanes 5 and 6**), orf19.5962_5DR/orf19.5962_3DR (**lanes 7 and 8**), and either plasmid DNA pBME101 as template (**lanes 4, 6 and 8**) or without template DNA (**lanes 3, 5 and 7**). Lane 2 was a template but no primers control reaction. Lanes 1 and 9 contained the $1Kb^{+}$ ladder as size standards.

APPENDIX 6: STRATEGY FOR ORF DISRUPTION USING THE *UAU1* CASSETTE



The *UAU1* cassette comprises an intact *ARG4* gene flanked by *URA3* segments that are non-functional, so the *UAU1* cassette confers an Arg+ Ura- phenotype. The *URA3* segments share 530 bp of homology and can thus recombine to yield an intact *URA3* gene. Recombination excises the *ARG4* gene and results in an Arg+ Ura+ phenotype.

One copy of the target ORF is disrupted with a *UAU1* insertion through transformation with selection for an Arg+ phenotype **(Panel A and B)**. Growth of the Arg+ transformant yields rare recombinant segregants in which the *UAU1* insertion is homozygous **(Panel C)**. They can undergo recombinational excision to yield a unique Arg+ Ura+ phenotype **(Panel D)**. Alternatively, growth of the Arg+ transformant may yield segregants in which trisomy, translocation, tandem duplication, or other genetic rearrangements produce an Arg+ Ura+ phenotype but retain a functional copy of the target ORF **(not shown)**.