

Niche-specific regulation of central metabolic pathways in a fungal pathogen

OnlineOpen: This article is available free online at www.blackwell-synergy.com

Caroline J. Barelle, Claire L. Priest,
Donna M. MacCallum, Neil A. R. Gow,
Frank C. Odds and Alistair J. P. Brown*

School of Medical Sciences, University of Aberdeen,
Foresterhill, Aberdeen AB25 2ZD, UK.

Summary

To establish an infection, the pathogen *Candida albicans* must assimilate carbon and grow in its mammalian host. This fungus assimilates six-carbon compounds via the glycolytic pathway, and two-carbon compounds via the glyoxylate cycle and gluconeogenesis. We address a paradox regarding the roles of these central metabolic pathways in *C. albicans* pathogenesis: the glyoxylate cycle is apparently required for virulence although glyoxylate cycle genes are repressed by glucose at concentrations present in the bloodstream. Using GFP fusions, we confirm that glyoxylate cycle and gluconeogenic genes in *C. albicans* are repressed by physiologically relevant concentrations of glucose, and show that these genes are inactive in the majority of fungal cells infecting the mouse kidney. However, these pathways are induced following phagocytosis by macrophages or neutrophils. In contrast, glycolytic genes are not induced following phagocytosis and are expressed in infected kidney. Mutations in all three pathways attenuate the virulence of this fungus, highlighting the importance of central carbon metabolism for the establishment of *C. albicans* infections. We conclude that *C. albicans* displays a metabolic program whereby the glyoxylate cycle and gluconeogenesis are activated early, when the pathogen is phagocytosed by host cells, while the subsequent progression of systemic disease is dependent upon glycolysis.

Introduction

Candida albicans is the major systemic fungal pathogen of humans. This fungus exists as a relatively harmless

commensal in the oral cavity and gastrointestinal tracts of most individuals, but when the defences of the host become compromised, it can cause mucocutaneous infections such as oral or vaginal candidiasis (Odds, 1988; Calderone, 2002). In severely immunocompromised individuals, *C. albicans* can establish deep-seated systemic infections, which in some patient groups are often fatal (Odds, 1988; Calderone, 2002). Bloodstream infections are thought to arise by two major routes: through penetration of mucosal surfaces, or via intravascular catheters (Velasco *et al.*, 2000; Kullberg and Oude Lashof, 2002), and the formation of *C. albicans* biofilms upon catheters can exacerbate the infection source (Douglas, 2003). In the absence of effective immune defences, *C. albicans* can then disseminate via the bloodstream and colonize internal organs such as the kidney.

To grow, a microbe must assimilate carbon. Pathogens such as *C. albicans*, which can thrive within diverse niches such as the skin, mucous membranes, blood and internal organs of its human host and in biofilms (Odds, 1988; Calderone, 2002), must display sufficient metabolic flexibility to assimilate the available nutrients in these niches. Data from several transcript profiling studies are consistent with this notion (reviewed in Brown, 2005). For example, amino acid biosynthetic genes are upregulated in *C. albicans* cells growing in biofilms (Garcia-Sanchez *et al.*, 2005). Also, following exposure to human neutrophils or cultured macrophages, *C. albicans* populations upregulate amino acid biosynthetic genes and display a shift from fermentative to non-fermentative metabolism (Rubin-Bejerano *et al.*, 2003; Lorenz *et al.*, 2004; Fradin *et al.*, 2005). This includes the downregulation of glycolytic genes and the activation of glyoxylate cycle genes (*ICL1*, *MLS1*), which facilitate the assimilation of two-carbon compounds in concert with gluconeogenic genes (*PCK1*, *FBP1*). Further evidence for the activation of glyoxylate cycle genes following exposure to macrophages has been obtained by differential display reverse transcription polymerase chain reaction (PCR) (Prigneau *et al.*, 2003).

Lorenz and Fink (2001) have shown that *C. albicans* *icl1/icl1* mutants display attenuated virulence in the mouse model of systemic candidiasis. This has led to the suggestion that the glyoxylate cycle is required for fungal virulence (Lorenz and Fink, 2001; 2002). However in *Saccharomyces cerevisiae*, gluconeogenic and glyoxylate

Received 30 August, 2005; revised 14 November, 2005; accepted 22 November, 2005. *For correspondence. E-mail al.brown@abdn.ac.uk; Tel. (+44) 1224 555883; Fax (+44) 1224 555844.

cycle genes are exquisitely sensitive to glucose (Yin *et al.*, 2003). These genes are repressed by glucose at concentrations as low as 0.01%, which are well below those present in the bloodstream (3–5 mM, which is equivalent to about 0.06–0.1% glucose). If *C. albicans* gluconeogenic and glyoxylate cycle genes are regulated in an analogous fashion to those in *S. cerevisiae*, which is often presumed to be the case, one would expect them to be repressed during systemic infections. How then is the glyoxylate cycle required for the establishment of systemic *C. albicans* infections?

To address this apparent paradox, we have revisited the role of the glyoxylate cycle in *C. albicans* virulence. Furthermore, we have extended this analysis to examine the glycolytic and gluconeogenic pathways. In this study we used GFP fusions, rather than transcript profiling, to monitor gene activity (Barelle *et al.*, 2004). This was done for three main reasons. First, transcript profiling averages the behaviour of potentially heterogeneous cell populations, whereas the use of GFP fusions has allowed us to examine the behaviour of individual *C. albicans* cells within complex niches. Second, this approach has allowed us to extend our analyses beyond the *in vitro* and *ex vivo* models that have been used for transcript profiling, to monitor gene expression levels in the mouse model of systemic candidiasis. Third, while transcript profiling has indicated whether genes are up- or downregulated (by generating expression ratios), our GFP approach has revealed interesting differences in the absolute expression levels for specific gene fusions. All three factors have proved important in describing the behaviour of this pathogenic fungus *in vivo*. Taken together with our phenotypic analysis of metabolic null mutants, our GFP studies suggest that *C. albicans* differentially regulates its central pathways of carbon assimilation at different stages and sites of disseminated infections.

Results

Regulation of glycolytic, gluconeogenic and glyoxylate cycle genes *in vitro*

Promoter–GFP fusions were constructed to monitor the regulation of glycolytic, gluconeogenic and glyoxylate cycle genes in *C. albicans* (Fig. 1). Most of the enzymes on the glycolytic pathway catalyse reversible reactions that also contribute to gluconeogenesis. However, two steps in glycolysis are essentially irreversible and these are catalysed by the glycolysis-specific enzymes, phosphofruktokinase and pyruvate kinase. *PFK2* encodes one of two phosphofruktokinase subunits, and *PYK1* encodes pyruvate kinase. *PCK1* encodes the gluconeogenic-specific enzyme, phosphoenolpyruvate carboxykinase. *ICL1* encodes the glyoxylate cycle enzyme, isocitrate lyase (Fig. 1). Therefore, the *PFK2*, *PYK1*, *PCK1* and

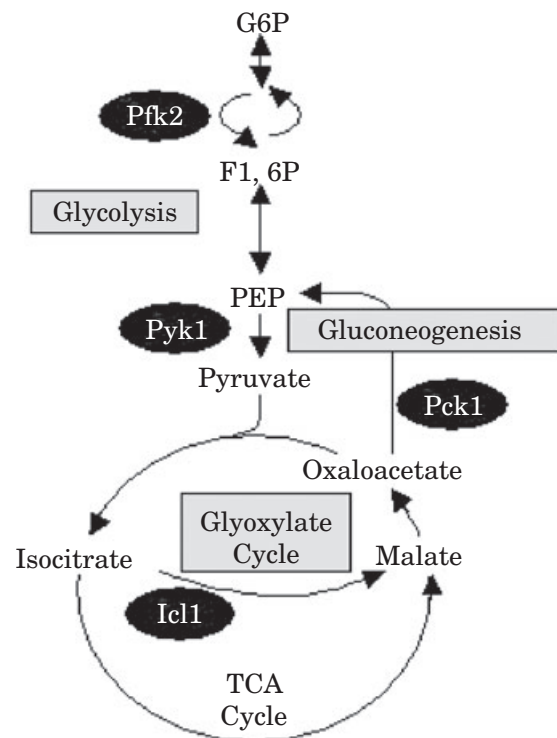


Fig. 1. Cartoon of central carbon metabolism, highlighting the steps analysed in this study.

ICL1 promoter regions were cloned upstream of the yeast enhanced GFP (yEGFP) gene in the vector pGFP, and these plasmids were stably integrated at the *RPS1* locus in the *C. albicans* genome. The genotype of these strains was confirmed by diagnostic PCR and Southern blotting (not shown).

The expression patterns of these promoter fusions were first examined *in vitro*. To achieve this, we grew the *C. albicans* strains on different carbon sources and measured the mean GFP fluorescence per cell. As expected (Leuker *et al.*, 1997; Lorenz and Fink, 2001; Murad *et al.*, 2001), the *PCK1*- and *ICL1*-GFP fusions were expressed during growth on amino acids and repressed during growth on glucose (Fig. 2A). The *PCK1* and *ICL1* fusions displayed similar expression patterns in yeast and hyphal cells (not shown). Interestingly, these *C. albicans* genes were sensitive to low glucose concentrations, like their homologues in *S. cerevisiae* (Yin *et al.*, 2003). Significantly, the *PCK1* and *ICL1* fusions were repressed at physiologically relevant glucose concentrations (0.1%), implying that the gluconeogenic pathway and the glyoxylate cycle are inactive under these conditions (Fig. 2B).

The *PFK2*- and *PYK1*-GFP gene fusions, which were designed to monitor glycolytic activity, were expressed at low levels following protracted growth on non-fermentative growth media containing amino acids as sole carbon

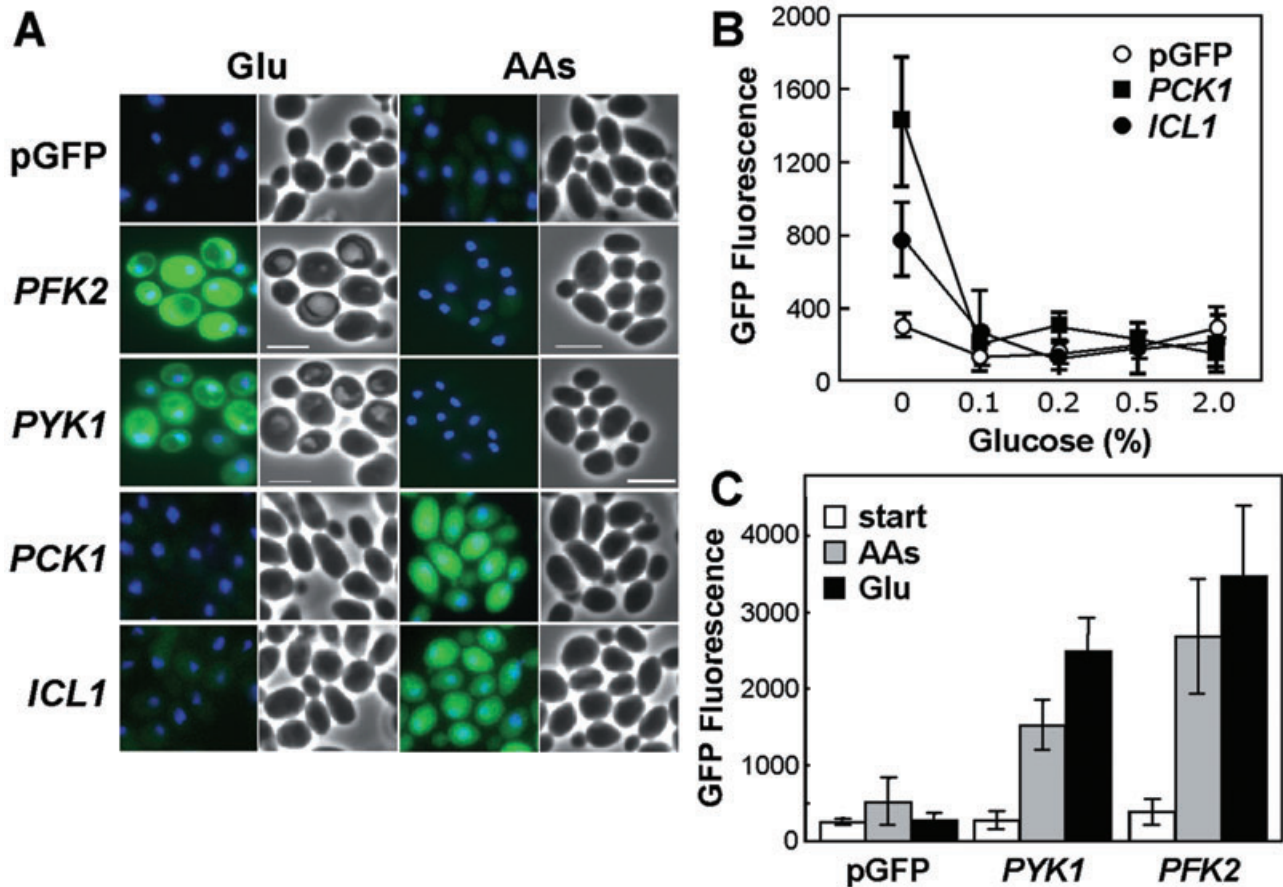


Fig. 2. Differential *in vitro* regulation of glycolysis, gluconeogenesis and the glyoxylate cycle in *C. albicans*. A. *C. albicans* strains containing *PFK2*-, *PYK1*-, *PCK1*- and *ICL1*-GFP promoter fusions or the empty pGFP control (CJB-1, CJB-2, CJB-3, CLM1-1, CLM3-2; Table 1) were examined after growth overnight on minimal media containing 2% glucose (Glu) or 2% casamino acids (AAs) as sole carbon source. Merged GFP and DAPI images are shown alongside the corresponding light micrographs. Scale bar represents 10 μ m. B. Repression of *PCK1*- and *ICL1*-GFP by different concentrations of glucose (mean GFP fluorescence intensity per cell). C. Quantification of mean GFP fluorescence levels for *C. albicans* cells with *PFK2*- and *PYK1*-GFP fusions resuming growth on glucose or amino acids: cells grown overnight on amino acids (start); cells grown for 2 h on glucose (Glu) or amino acids (AAs).

source (Fig. 2A). However, both fusions were activated when *C. albicans* cells resumed growth after stationary phase, irrespective of whether amino acids or glucose was available as sole carbon source (Fig. 2C). The *PFK2* and *PYK1* fusions displayed similar expression patterns in yeast and hyphal cells (not shown). We conclude that *PFK2*- and *PYK1*-GFP expression reflects active growth of *C. albicans* rather than glycolytic activity *per se*.

To test whether the behaviour of these promoter fusions reflected the expression of the native proteins, we examined the expression levels of the Pfk2, Pyk1, Pck1 and Icl1 proteins during the growth of *C. albicans* in the presence or absence of glucose. This was done as part of a global proteomics study of *C. albicans* (in preparation: <http://www.abdn.ac.uk/cogeme>). The glycolytic enzymes, Pfk2 and Pyk1, were expressed at roughly equivalent levels during exponential growth in the presence or absence of glucose (not shown). In contrast, the expres-

sion of the Pck1 and Icl1 proteins was repressed by glucose. These proteins were not detected during growth on glucose. Also, Pck1 expression levels decreased about threefold within 2 h of 0.1% glucose addition to cells growing on amino acids or lactate as sole carbon source (not shown). Therefore, the behaviour of the *PFK2*-, *PYK1*-, *PCK1*- and *ICL1*-GFP fusions accurately reflected the expression of the native enzymes.

Differential expression of glycolytic, gluconeogenic and glyoxylate cycle genes in *ex vivo* models

Having established the expression patterns of the *PYK1*, *PFK2*, *PCK1* and *ICL1* fusions *in vitro*, we examined their behaviour in two medically significant *ex vivo* models. Neutrophils and macrophages represent first lines of innate host defence when *C. albicans* cells infect the bloodstream or endothelia respectively. Neutropenic patients are

especially susceptible to bloodstream candidaemias (Abi-Said *et al.*, 1997). Transcript profiling has revealed that glyoxylate cycle genes are induced in *C. albicans* cells following exposure to neutrophils or macrophages (Lorenz *et al.*, 2004; Fradin *et al.*, 2005). Therefore, we profiled single *C. albicans* cells under similar conditions (Fig. 3). The *C. albicans* strains were exposed to primary human neutrophils or cultured murine J774A-1 macrophages, and the expression in phagocytosed fungal cells was compared with the expression in non-phagocytosed cells (ratio of mean GFP fluorescence per fungal cell).

Three important observations were made. First, the induction of *ICL1*- and *PCK1*-GFP was dependent upon the internalization of fungal cells by neutrophils or macrophages, and was not observed in non-phagocytosed cells. In contrast, the glycolytic gene fusions were expressed both inside and outside the neutrophils and macrophages, and no significant induction was observed following phagocytosis. These observations are consistent with transcript profiling experiments which have suggested that glyoxylate cycle and gluconeogenic genes are induced following phagocytosis. However, these studies were unable to distinguish unambiguously between phagocytosed and non-phagocytosed cells within heterogeneous populations (Lorenz *et al.*, 2004; Fradin *et al.*, 2005). Second, GFP expression levels were generally an order of magnitude higher following phagocytosis by neutrophils, compared with macrophages (Fig. 3). This difference is probably attributable to differences in transcription because *in vitro* experiments have shown that GFP stability remains unaffected following exposure of *C. albicans* cells to an oxidative stress (5 mM H₂O₂) or low pH (pH 4) (not shown). These data add to the available transcript profiling data, which did not provide information about absolute expression levels in phagocytosed cells (Lorenz *et al.*, 2004; Fradin *et al.*, 2005). Our data are consistent with the idea that genes involved in central carbon metabolism are more active in *C. albicans* cells engulfed by neutrophils, although neutrophils are thought to be more potent than macrophages at killing fungal cells (Fradin *et al.*, 2005). Third, *PCK1*-GFP expression levels were reproducibly lower than *ICL1*-GFP expression levels after phagocytosis by neutrophils (Fig. 3A). This suggests that glyoxylate cycle activity might not always be associated with gluconeogenesis. It is possible that, in some body sites, *C. albicans* cells might exploit the glyoxylate cycle for the generation of metabolic intermediates rather than hexose anabolism.

Differential expression of glycolytic, gluconeogenic and glyoxylate cycle genes during systemic infections

Having characterized the behaviour of the *PFK2*-, *PYK1*-, *PCK1*- and *ICL1*-GFP fusions *in vitro* and *ex vivo*, we

analysed their expression *in vivo* in the mouse model of disseminated *Candida* infection (Fig. 4). The *C. albicans* strains were used to establish infections in immunocompetent mice, and the GFP fluorescence examined in the infected kidneys, as described previously (Barelle *et al.*, 2004). The *PFK2*- and *PYK1*-GFP fusions were expressed in nearly all of the fungal cells infecting these kidneys, indicating that most *C. albicans* cells were actively growing in the infected tissue, or at least had been growing shortly before the tissue was harvested and fixed. In contrast, of the thousands of fungal cells we examined in infected kidneys, only about half expressed *ICL1*-GFP, and about one-third expressed *PCK1*-GFP. Two significant conclusions can be drawn from these observations. First, it is clear that the population of fungal cells infecting the kidney is highly heterogeneous, suggesting that individual fungal cells occupy subtly different micro-environments and are exposed to different environmental signals within the kidney. Second, only about third of *C. albicans* cells infecting the kidney appear to assimilate carbon via gluconeogenesis. The analysis of the *PFK2*- and *PYK1*-GFP fusions suggested that these cells were actively growing (or had been very recently). Therefore, by inference, most *C. albicans* cells growing in the kidney were assimilating carbon via glycolytic metabolism. This did not seem consistent with the observation that the glyoxylate cycle is required for *C. albicans* virulence (Lorenz and Fink, 2001).

Virulence of C. albicans null mutants with blocks in glycolysis, gluconeogenesis or the glyoxylate cycle

Lorenz and Fink (2001) reported that *C. albicans icl1/icl1* mutants display attenuated virulence in the mouse model of systemic candidiasis. However, after their article was published it was shown that *URA3* position effects can influence the outcome of such virulence assays (Sundstrom *et al.*, 2002; Brand *et al.*, 2004; Sharkey *et al.*, 2005). As the *URA3* marker was used to generate the *icl1/icl1* mutant, it was conceivable that the virulence of this mutant was attenuated by the position of the *URA3* at the *icl1* locus, rather than the disruption of the *ICL1* gene itself. Therefore, we re-evaluated the effect of inactivating *ICL1* upon *C. albicans* virulence. Having generated a homozygous *icl1/icl1* mutant in *C. albicans* RM1000 by standard procedures (Wilson *et al.*, 1999), we reintroduced the *URA3* and *HIS1* markers back into the mutant through integration at the *RPS1* locus via the vector, Clp20. Clp20 is derived from Clp10 (Dennison *et al.*, 2005), which has been shown to circumvent *URA3* marker position effects (Brand *et al.*, 2004). In addition, we extended this work by generating congenic *pck1/pck1* and *pyk1/pyk1* null mutants with a view to determining the impact of these mutations upon *C. albicans* virulence.

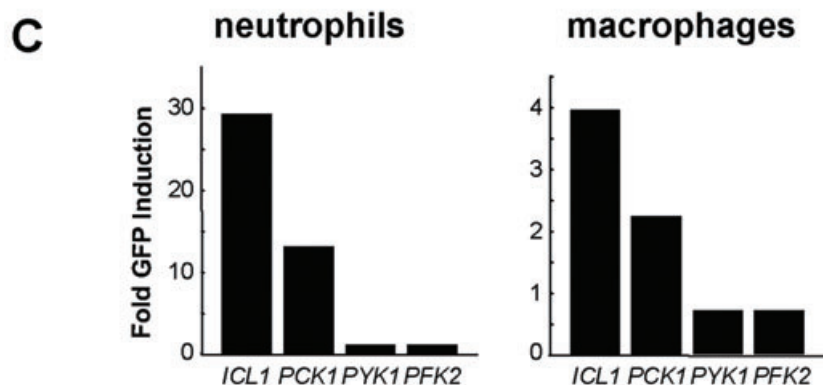
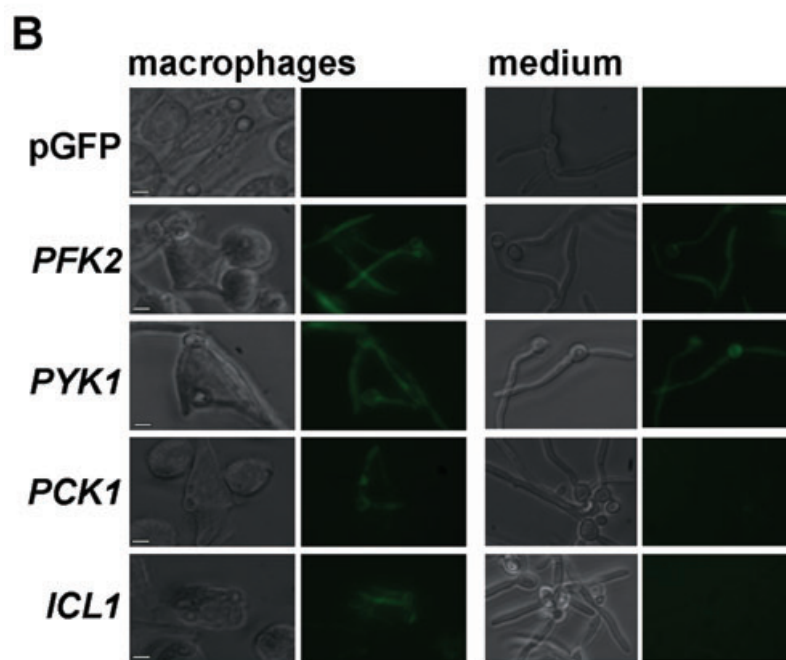
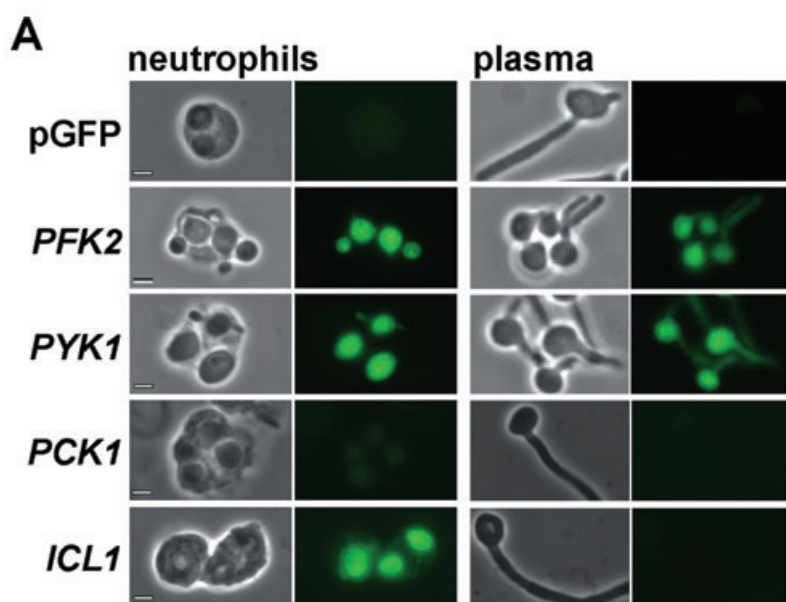


Fig. 3. Differential regulation of *PFK2*-, *PYK1*-, *PCK1*- and *ICL1*-GFP fusions in *C. albicans* following phagocytosis by (A)neutrophils or (B) macrophages.

A. *C. albicans* cells were mixed with primary human neutrophils in a 1:1 ratio, and examined microscopically after 1.5 h. The ratio of GFP expression in phagocytosed to non-phagocytosed *C. albicans* cells was measured (C). Corresponding light and fluorescence micrographs of phagocytosed *C. albicans* cells (neutrophils), and light and fluorescence micrographs of control cells in plasma alone (plasma) are shown; bars represent 10 μ m. Fold induction was measured by comparing the mean fluorescence intensity for phagocytosed cells with that for non-phagocytosed *C. albicans* cells ($n > 50$). *C. albicans* strains containing *PFK2*-, *PYK1*-, *PCK1*- or *ICL1*-GFP fusions were compared with the control carrying the empty vector, pGFP (CJB-1, CJB-2, CJB-3, CLM1-1, CLM3-2: Table 1).

B. Cultured murine J774A-1 macrophages were mixed with *C. albicans* cells in a 1:1 ratio and analysed after 3 h, as described in A: phagocytosed *C. albicans* cells (macrophages); non-phagocytosed cells (medium). Scale bar represents 20 μ m.

C. Fold induction of GFP fluorescence in phagocytosed cells versus non-phagocytosed cells in neutrophils and macrophages respectively.

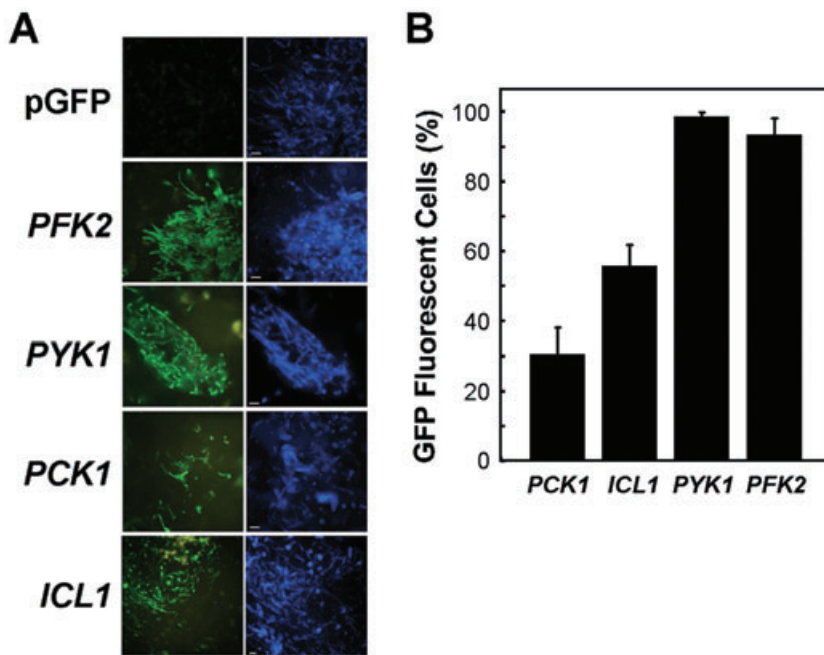


Fig. 4. Differential regulation of *PFK2*-, *PYK1*-, *PCK1*- and *ICL1*-GFP fusions in *C. albicans* in the kidney during systemic candidiasis.

A. Corresponding GFP (green) and Calcofluor White-stained (blue) images of *C. albicans* cells infecting the mouse kidney. *C. albicans* strains containing *PFK2*-, *PYK1*-, *PCK1*- or *ICL1*-GFP fusions were compared with the control strain carrying the empty vector, pGFP (CJB-1, CJB-2, CJB-3, CLM1-1, CLM3-2; Table 1).

B. Proportion of *C. albicans* cells ($n > 1000$) infecting the kidney that display GFP fluorescence above background levels, in animals displaying clinical signs of infection.

The growth phenotype of each mutant was examined *in vitro* (Fig. 5). As expected the *pyk1/pyk1* mutant did not grow on glucose, but grew on non-fermentable carbon sources. The *pck1/pck1* mutant displayed growth defects on non-fermentable carbon sources, but grew on glucose. As expected (Lorenz and Fink, 2001), the *icl1/icl1* mutant did not grow on acetate, but grew on the other carbon sources. These phenotypes were suppressed by the reintroduction of the corresponding wild-type gene (Fig. 5).

The virulence of these *C. albicans* mutants and the control reintegrant strains was then tested in the mouse model of systemic candidiasis (MacCallum and Odds, 2005). The *icl1/icl1* and *pck1/pck1* cultures were prepared in NGY medium, which contains glucose. However, the *pyk1/pyk1* mutant did not grow on glucose (Fig. 5), and therefore this strain was grown on a derivative of NGY medium containing amino acids instead of glucose. Control experiments, in which the parental and reintegrant (*pyk1/pyk1*/*PYK1*) strains were prepared under identical conditions, confirmed that this change had a minimal effect upon the virulence of *C. albicans* (Fig. 6).

Several observations were made in the virulence assays. First, the inactivation of *ICL1* did attenuate the virulence of *C. albicans*, albeit partially, and this defect was suppressed by the reintroduction of the *ICL1* gene (Fig. 6A). This confirms the original study of Lorenz and Fink (2001), which showed that the glyoxylate cycle (or at least the *ICL1* gene itself) is required for normal levels of virulence in *C. albicans*. Second, the *C. albicans* *pck1/pck1* and *pyk1/pyk1* mutants also displayed attenuated virulence, and these defects were suppressed by the reintroduction of the wild-type *PCK1* or *PYK1* genes respec-

tively (Fig. 6A). Hence these glycolytic and gluconeogenic genes are also required for the virulence of *C. albicans*. Third, there was a strong correlation between the virulence data and the corresponding fungal burdens observed in the tissues of infected animals (Fig. 6B). This lends weight to conclusions relating to the relative effects of the gene knockouts upon virulence. Fourth, the degree of virulence attenuation was significantly greater for *pyk1/pyk1* cells than for *icl1/icl1* or *pck1/pck1* cells. The inacti-

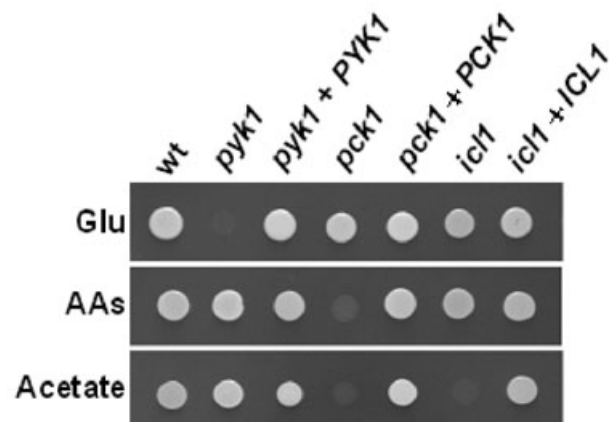


Fig. 5. Growth of *C. albicans* *pyk1/pyk1*, *pck1/pck1* and *icl1/icl1* null mutants on different carbon sources. *C. albicans* wild type, *pyk1/pyk1*, *pck1/pck1* and *icl1/icl1* strains (wt, CLM19-3; *pyk1*, CLM44-5; *pck1*, CLM56-4; *icl1*, CLM25-5; Table 1) and the corresponding control strains containing a reintegrated copy of the wild-type gene (*pyk1* + *PYK1*, CLM45-9; *pck1* + *PCK1*, CLM57-2; *icl1* + *ICL1*, CLM26-1; Table 1) were grown on minimal medium containing 2% glucose (Glu), 2% amino acids (AAs) or 2% acetate as sole carbon source.

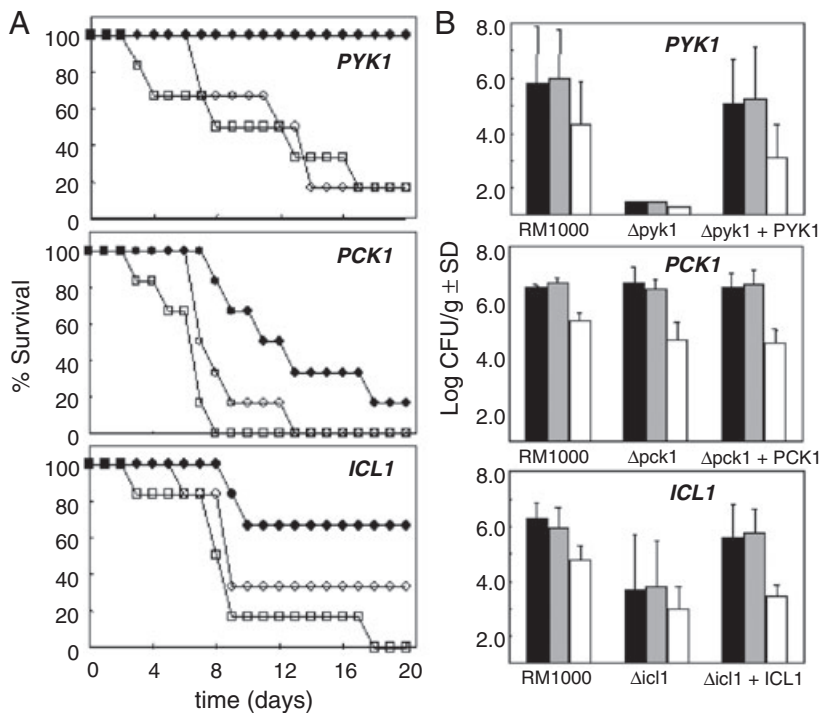


Fig. 6. Virulence of *C. albicans* *pyk1/pyk1*, *pck1/pck1* and *icl1/icl1* null mutants in the mouse model of systemic infection.

A. Survival of immunocompetent female BALB/c mice following tail vein injection of *C. albicans* cells: open squares, first control representing the parental strain, RM1000 containing Clp20 (CLM19-3; Table 1); closed circles, experiment representing the homozygous null mutant containing Clp20 (*icl1/icl1*, CLM25-5; *pck1/pck1*, CLM56-4; *pyk1/pyk1*, CLM44-5; Table 1); open circles, second control representing the null mutant containing Clp20 and the wild-type gene (*ICL1*, CLM26-1; *PCK1*, CLM57-2; *PYK1*, CLM45-9; Table 1).

B. Tissue burdens in mouse organs infected with the same *C. albicans* strains. Fungal colony forming units per gram of tissue were measured in the left kidney (black), right kidney (grey) and brain (white).

vation of *PCK1* or *ICL1* did attenuate virulence (Fig. 6A), but did not prevent fungal growth in the host (Fig. 6B). We conclude that neither gluconeogenesis nor the glyoxylate cycle is essential for *C. albicans* to thrive in the host. However, glycolysis is important for the development of a systemic infection.

Discussion

Our data confirm and reinforce previous observations relating to the role of the glyoxylate cycle, and the *ICL1* gene in particular, in *C. albicans* pathogenesis. However, we have significantly extended these observations by describing the relative contributions of glycolysis and gluconeogenesis to the development of systemic candidiasis, and by providing significant insights into the heterogeneous behaviour of *C. albicans* cells within complex micro-environments in its mammalian host.

Several transcript profiling studies have indicated that *C. albicans* glyoxylate cycle genes (*ICL1*, *MLS1*) are induced following exposure to host defences (Fradin *et al.*, 2003; 2005; Lorenz *et al.*, 2004). This was the case following exposure to macrophages, which play an important defensive role during infections of parenchyma, and to neutrophils, which kill fungal cells present in the bloodstream. As a large proportion of *C. albicans* cells in these samples had been phagocytosed by the mammalian cells, it was presumed that the fungal glyoxylate cycle genes were induced after phagocytosis rather than in response to cell-cell contact. Our data support the view that these

genes are induced after phagocytosis, when the fungal cells enter an environment relatively free of glucose (Fig. 3).

The inactivation of *ICL1* has been reported to be required for fungal virulence (Lorenz and Fink, 2001). However, *C. albicans* *ICL1* was strongly repressed in response to low levels of glucose (0.1%: Fig. 2) that are equivalent to those found in the bloodstream in humans and mice. Furthermore, although the gene is induced following phagocytosis (Fig. 3), it is not expressed in most *C. albicans* cells infecting the kidney (Fig. 4). How then can *ICL1* be required for the development of systemic infections? Several possible explanations might account for this apparent paradox. First, the interpretation of the *C. albicans* virulence assays performed by Lorenz and Fink (2001) might have been compromised by *URA3* position effects that were described after their findings were published (Sundstrom *et al.*, 2002; Brand *et al.*, 2004; Sharkey *et al.*, 2005). We have shown that this is not the case by testing the virulence a new homozygous *icl1/icl1* mutant alongside appropriate controls that excluded potential *URA3* position effects (Fig. 4). Second, *ICL1* might contribute to the virulence of *C. albicans* without being essential for the establishment of systemic infections. We show that this is the case. *ICL1* is required for normal levels of *C. albicans* virulence. However, the virulence of *C. albicans* *icl1/icl1* cells is only partially attenuated, and these cells are capable of establishing lethal systemic infections, albeit more slowly than wild-type cells (Fig. 6).

How might *ICL1* contribute to *C. albicans* virulence without being essential for the establishment of systemic infections? The transcript profiling and GFP data indicate that *ICL1* is induced following phagocytosis (Fig. 3: Lorenz *et al.*, 2004; Fradin *et al.*, 2005). Therefore, the glyoxylate cycle might help to protect *C. albicans* against host antimicrobial defences by facilitating anabolic metabolism in the absence of fermentable carbon sources (Lorenz and Fink, 2002). The absence of a functional glyoxylate cycle might lead to a reduction in the number of viable fungal cells that are capable of colonizing host tissues. Also, *ICL1* might contribute to the growth of some fungal cells at infection sites (Fig. 4).

The same rationale might apply to the *PCK1* gene and gluconeogenesis. Like *ICL1*, *PCK1* was induced following phagocytosis (Fig. 3: Lorenz *et al.*, 2004; Fradin *et al.*, 2005). Like the *C. albicans icl1/icl1* strain, the virulence of the *pck1/pck1* mutant was partially attenuated in the mouse model of systemic candidiasis. *C. albicans pck1/pck1* cells are unable to generate the five and six carbon sugars required for the biosynthesis of cell wall and nucleic acids, for example, from two or three carbon substrates (Fig. 1), and hence were unable to grow on such carbon sources (Fig. 5). Therefore, the fact that the *icl1/icl1* and *pck1/pck1* mutants are able to colonize kidney tissue (Fig. 6) indicates that, while growing *in vivo*, *C. albicans* depends mainly upon the assimilation of fermentable carbon sources *in vivo*, such as blood glucose for example. This idea is reinforced by the observation that *pyk1/pyk1* cells are avirulent (Fig. 6), indicating that *C. albicans* depends upon glycolysis for growth in the host. Hence in principle, some drugs that inhibit fungal glycolytic enzymes without affecting their mammalian counterparts could prove to be effective antifungal agents.

Our analyses of GFP fusions have provided direct evidence for a high degree of metabolic heterogeneity within fungal cell populations *in vivo* (Fig. 4). Almost all *C. albicans* cells infecting the mouse kidney expressed the *PFK2*- and *PYK1*-GFP fusions, indicating that most of these fungal cells were actively growing, or had recently been actively growing. [The half-life of GFP in growing *C. albicans* is about 1.5 h (Barelle *et al.*, 2004)] However, fewer than half of these cells expressed the *PCK1*- and *ICL1*-GFP fusions (Fig. 4). This implies that, while most cells were assimilating carbon via the glycolytic pathway, some had switched to gluconeogenic metabolism. Therefore, glucose appears to become limiting in some micro-environments within the infected kidney. Currently methods are being developed for the transcript profiling of *C. albicans* cells isolated from infected tissues. The heterogeneity of fungal populations within foci of infection has major implications for the interpretation of these transcript profiling data sets.

In conclusion, our data reveal that the pathogen *C. albicans* regulates central carbon metabolism in a niche-specific manner during disease establishment and progression. During the early stages of a systemic infection, *C. albicans* activates the glyoxylate cycle and gluconeogenesis in response to phagocytosis. In the latter stages of an infection, when the fungus is colonizing tissue, glycolytic metabolism predominates. Presumably, this increases the biological fitness of this pathogen within its host.

Experimental procedures

Strains and growth conditions

Candida albicans strains (Table 1) were grown in YPD or synthetic complete medium (Sherman, 1991; Kaiser *et al.*, 1994) or in minimal medium (0.67% Yeast Nitrogen Base) containing 2% glucose, 2% casamino acids or 2% acetate as sole carbon source.

Strain construction

The plasmids pGFP and pPCK1-GFP have been described previously (Barelle *et al.*, 2004). To generate pICL1-GFP, the *CalICL1* promoter region (−983 to −1, relative to the start codon) was PCR-amplified using the primers ICL1-5' and ICL1-3', and cloned between the XhoI and HindIII sites of pGFP. To make pPYK1-GFP, the *CaPYK1* promoter region (−1576 to −3) was PCR-amplified using the primers PYK5'-2 and PYK3'-3, and cloned between the BstEII and MluI sites of pGFP. To produce pPFK2-GFP, the *CaPFK2* promoter region (−992 to −1) was PCR-amplified using primers PFK2-5' and PFK2-3' and cloned between the XhoI and HindIII sites of pGFP. These plasmids were linearized with StuI and integrated at the *RPS1* locus in *C. albicans* CAI4 (Murad *et al.*, 2000). The genotypes of transformants were confirmed by PCR and Southern blotting, as described previously (Barelle *et al.*, 2004).

The *C. albicans pyk1/pyk1* null mutant was constructed using the parental strain RM1000 (Table 1). Each *PYK1* allele was inactivated sequentially with *loxP*-*URA3*-*loxP* (*LUL*) and *loxP*-*HIS1*-*loxP* (*LHL*) disruption cassettes (Dennison *et al.*, 2005) generated by PCR amplification with the oligonucleotide primers *PYK*-L1 and *PYK*-L2 (Table 2). The *pyk1::LUL* and *pyk1::LHL* disruption cassettes contained about 65 bp of homology to the 5' and 3' ends of the *PYK1* locus, and deleted codons 19–490 of the *PYK1* open reading frame. The *pyk1::LUL* cassette was transformed into RM1000, transformants selected on the basis of their uridine protrophy, and integration at the correct locus confirmed by PCR diagnosis with the primers *PYK*-5'-DIAG and *URA3*-REV-DIAG (Table 2), as described previously (Wilson *et al.*, 1999). The resultant heterozygote (*pyk1::loxP*-*URA3*-*loxP*/*PYK1*: PD24) was then transformed with the *pyk1::LHL* cassette, and the introduction of this allele and the removal of the last wild-type *PYK1* allele from the *C. albicans* genome was confirmed by PCR using the primer *PYK*-5'-DIAG with *HIS1*-REV-DIAG or *PYK*-IP, respectively (Table 2), thereby generating the homozygous null mutant, CLM5-1 (Table 1).

Table 1. *C. albicans* strains.

Strain	Genotype	Parent	Source
SC5314	Wild type		Gillum <i>et al.</i> 1984
CAF2-1	<i>URA3/ura3::λ imm434</i>	SC5314	Fonzi and Irwin (1993)
CAI4	<i>ura3::λ imm434/ura3::λ imm434</i>	CAF2-1	Fonzi and Irwin (1993)
CJB-1	<i>ura3::λ imm434/ura3::λ imm434</i> , pGFP	CAI4	Barelle <i>et al.</i> (2004)
CJB-2	<i>ura3::λ imm434/ura3::λ imm434</i> , pPCK1-GFP	CAI4	Barelle <i>et al.</i> (2004)
CJB-3	<i>ura3::λ imm434/ura3::λ imm434</i> , pICL1-GFP	CAI4	This study
CLM1-1	<i>ura3::λ imm434/ura3::λ imm434</i> , pPYK1-GFP	CAI4	This study
CLM3-2	<i>ura3::λ imm434/ura3::λ imm434</i> , pPFK2-GFP	CAI4	This study
RM1000	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i>	CAI4	Wilson <i>et al.</i> (1999)
CLM19-3	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , Clp20 (<i>URA3</i> , <i>HIS1</i>)	RM1000	This study
CLM4-10	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>ICL1/icl1::URA3</i>	RM1000	This study
CLM18-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>icl1::HIS1/icl1::URA3</i>	RM1000	This study
CLM21-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>icl1::HIS1/icl1::ura3</i>	RM1000	This study
CLM25-5	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>icl1::HIS1/icl1::ura3</i> , Clp20 (<i>URA3</i> , <i>HIS1</i>)	RM1000	This study
CLM26-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>icl1::HIS1/icl1::ura3</i> , Clp20- <i>ICL1</i>	RM1000	This study
CLM17-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>PCK1/pck1::URA3</i>	RM1000	This study
CLM53-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pck1::HIS1/pck1::URA3</i>	RM1000	This study
CLM54-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pck1::HIS1/pck1::ura3</i>	RM1000	This study
CLM56-4	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pck1::HIS1/pck1::ura3</i> , Clp20 (<i>URA3</i> , <i>HIS1</i>)	RM1000	This study
CLM57-2	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pck1::HIS1/pck1::ura3</i> , Clp20- <i>PCK1</i>	RM1000	This study
PD24	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>PYK1/pyk1::URA3</i>	RM1000	This study
CLM5-1	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pyk1::HIS1/pyk1::URA3</i>	RM1000	This study
CLM29-2	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pyk1::HIS1/pyk1::ura3</i>	RM1000	This study
CLM44-5	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pyk1::HIS1/pyk1::ura3</i> , Clp20 (<i>URA3</i> , <i>HIS1</i>)	RM1000	This study
CLM45-9	<i>ura3::λ imm434/ura3::λ imm434</i> , <i>his1::hisG/his1::hisG</i> , <i>pyk1::HIS1/pyk1::ura3</i> , Clp20- <i>PYK1</i>	RM1000	This study

Table 2. Oligonucleotides.

Primer	Sequence (5' to 3')	Use
ICL1-5'	<u>CCCTCGAG</u> GTCTATGGAATCG	Cloning of <i>ICL1</i> promoter (XhoI site underlined)
ICL1-3'	GGAAGC <u>TTTTATTA</u> TGTTTATTC	Cloning of <i>ICL1</i> promoter (HindIII site underlined)
PYK5'-2	<u>CCCTCGAG</u> TATAGAAATGGGTTGC	Cloning of <i>PYK1</i> promoter (XhoI site underlined)
PYK3'-3	AAACTGCAGT <u>GATG</u> TAGTTGTAGG	Cloning of <i>PYK1</i> promoter (PstI site underlined)
PFK2-5'	<u>CCGCTCGAG</u> ATTCTATGACGTGGGT	Cloning of <i>PFK2</i> promoter (XhoI site underlined)
PFK2-3'	CCCAAGC <u>TTGGT</u> GGTTGTTTTTCGT	Cloning of <i>PFK2</i> promoter (HindIII site underlined)
ICL1-L1	TCCGGATGGCAATGTTCTTCTACTGCTTCCACTTCTAACGAACC ATCTCCAGATTTGGCTCCAGGGTTTTCCAGTCACG	PCR amplification of <i>icl1</i> disruption cassettes (homology to pLHL and pLUL plasmids underlined)
ICL1-L2B	CTTGATGTAAGTTTCTTGTTCTGCTCAGCTGGCATGGCCTTGTCCA GTTGAAAGATGGAGACTCACTAAAGGGAACAAAAGC	PCR amplification of <i>icl1</i> disruption cassettes (homology to pLHL and pLUL plasmids underlined)
ICL1-IP	TTCTGGGTTAGTGGCACCAA	Diagnosis of <i>icl1</i> alleles, internal 3' primer
ICL1-5'-DIAG	CAGGTGAAGAGTCACTTCTA	Diagnosis of <i>icl1</i> alleles, upstream primer
PCK-L1	AAATAATTATCAATCATGGCTCCTCTACTGCTGTTGAATCTTCAA TCAATTTCCGAGGTCACCCAGGGTTTTCCAGTCACG	PCR amplification of <i>pck1</i> disruption cassettes (homology to pLHL and pLUL plasmids underlined)
PCK1-L2	GACAATTCACCAGAGTGGATAGCATCCAAGATAGCTCTGGTGTGTA TTTCAATGGACATCTCACTAAAGGGAACAAAAGC	PCR amplification of <i>pck1</i> disruption cassettes (homology to pLHL and pLUL plasmids underlined)
PCK-5'-DIAG	GACATCACCTTTTTCAACC	Diagnosis of <i>pck1</i> alleles, internal 3' primer
PCK-IP	CAACAATACCGTCAACTC	Diagnosis of <i>pck1</i> alleles, upstream primer
PYK-L1	CTACATCAACAATGTCTCACTCATCTTTATCTTGGTTATCCAA CTTCAATGTTGAAACTGTTCCAGGGTTTTCCAGTCACG	PCR amplification of <i>pyk1</i> disruption cassettes (homology to pLHL and pLUL plasmids underlined)
PYK-L2	ATCAAGGCTTCTTTCCACTTAAGCTTGGACGATTCTAACAGTG TTAGATGACCAGAACCTCACTAAAGGGAACAAAAGC	PCR amplification of <i>pyk1</i> disruption cassettes (homology to pLHL and pLUL plasmids underlined)
PYK-IP	TCCTTACCTTCTTACC	Diagnosis of <i>pyk1</i> alleles, internal 3' primer
PYK-5'-DIAG	TTAGAAAGGAAACAAAAGG	Diagnosis of <i>pyk1</i> alleles, upstream primer
HIS1-REV-DIAG	CACAAGAAGCCTCAACGG	Diagnosis of <i>LHL</i> alleles, downstream primer
URA3-REV-DIAG	TAGTGTTACGAATCAATGGC	Diagnosis of <i>LUL</i> alleles, downstream primer
ICL1-Clp20-5'	<u>CCATCGAT</u> CAAAAACCTAATTTAACCCAGCG	Cloning of <i>ICL1</i> locus (ClaI site underlined)
ICL1-Clp20-3'	<u>CGACGCGT</u> TAAAGGCTCAAATTTGTTCCCG	Cloning of <i>ICL1</i> locus (MluI site underlined)
PYK-Clp20-5'	<u>CCATCGAT</u> TATAGAAATTTGGGTTGC	Cloning of <i>PYK1</i> locus (ClaI site underlined)
PYK-Clp20-3'	<u>CGACGCGT</u> GCAGAAATTAACGTATG	Cloning of <i>PYK1</i> locus (MluI site underlined)
PCK-Clp20-5'	<u>CCATCGAT</u> TTGGTCCATTGCCGT	Cloning of <i>PCK1</i> locus (ClaI site underlined)
PCK-Clp20-3'	<u>CGACGCGT</u> CCATAGGATTATGAGAC	Cloning of <i>PCK1</i> locus (MluI site underlined)

To generate the prototrophic reintegrant strain CLM45-9 (*pyk1/pyk1/PYK1*), *ura3⁻* segregants were selected on YPD containing 1 mg ml⁻¹ 5-fluoroorotic acid (Fonzi and Irwin, 1993), generating the strain CLM29-2. The plasmid Clp20-*PYK1* was then transformed into CLM29-2, selecting for the *URA3* marker, to create the strain CLM45-9. Clp20-*PYK1* contained the *PYK1* locus from coordinates -1576 upstream from the start codon to +354 downstream from the stop codon, and was generated using primers *PYK-Clp20-5'* and *PYK-Clp20-3'* (Table 2). As a control, the empty Clp20 vector was transformed into CLM29-2, to create CLM44-5 (Table 1). Clp20-based plasmids, which contain both *URA3* and *HIS1* markers, were integrated at the *RPS1* locus, and correct integration confirmed by PCR diagnosis (Dennison *et al.*, 2005).

Congenetic *C. albicans icl1/icl1* and *pck1/pck1* null mutants (CLM18-1 and CLM53-1, respectively; Table 1) were constructed using the same approach. In the *icl1/icl1* mutants, codons 121–430 of the *ICL1* open reading frame were deleted. In the *pck1/pck1* mutants, codons 18–459 of the *PCK1* open reading frame were deleted. Reintegrant and control strains were generated, as described above (Table 1). Clp20-*ICL1* contained the *ICL1* locus from -999 upstream from the start codon to +367 downstream from the stop codon, and Clp20-*PCK1* contained the *PCK1* locus from -977 to +500. The genotypes of all strains were confirmed by Southern blotting, as well as by PCR (Dennison *et al.*, 2005).

DNA manipulations

Cloning, PCR amplification and Southern analysis were performed as described previously (Sambrook *et al.*, 1989; Dennison *et al.*, 2005).

Proteomics

Protein extracts were prepared from mid-exponential *C. albicans* CLM19-3 cells (Table 1) growing on 2% glucose, casamino acids, lactate or oleic acid as sole carbon source. Extracts were also prepared 2 h after the addition of 0.1% glucose to cultures growing on 2% casamino acids, lactate or oleic acid. Protein extracts were subjected to 2D-gel electrophoresis, and the spots corresponding to the Pfk2, Pyk1, Pck1 and Icl1 proteins identified by peptide mass fingerprinting (Yin *et al.*, 2004). The relative level of each protein was measured by determining their mean spot volumes under each of the growth conditions in three independent replicate experiments.

Microscopy

Fungi were stained with Calcofluor White as described previously (Gow and Gooday, 1982). Nuclei were stained by overlaying mounting media containing DAPI (Vector Laboratories, Peterborough, UK). For phase contrast and fluorescence microscopy an Axioplan 2 microscope (Carl Zeiss, UK) was used with filter sets; XF 66, XF 67 and XF 77 (Omega Optical, Brattleboro, VT). Images were generated with a Hamamatsu CCD camera, and analysed with Openlab 3.0.9 software (Improvision, Coventry, UK). Relative GFP fluorescence intensities were quantified for individual yeast cells or hyphal compartments by measurement of regions of interest. Mean fluorescence intensities (\pm standard deviation) were then calculated for at least 50 indi-

vidual cells, as described before (Barelle *et al.*, 2004). GFP fluorescence levels never exceeded the quantitative range of the assay.

Ex vivo analysis of *C. albicans*

Neutrophils were isolated from human blood (Fradin *et al.*, 2005), *C. albicans* cells mixed with them in a 1:1 ratio, and GFP fluorescence measured after 1.5 h (Barelle *et al.*, 2004). Murine J774A-1 macrophages (Ralph *et al.*, 1975) were mixed with *C. albicans* cells in a 1:1 ratio and analysed after 3 h. The ratio of GFP expression in phagocytosed to non-phagocytosed *C. albicans* cells was measured. Fold induction was measured by comparison of mean fluorescence intensity for phagocytosed cells with that for non-phagocytosed *C. albicans* cells ($n > 50$).

Murine model of systemic candidiasis

Immunocompetent female BALB/c mice (Harlan Sera-lab, Loughborough, UK) were challenged intravenously with *C. albicans*. The *C. albicans icl1/icl1* and *pck1/pck1* strains and their controls (CLM19-3, CLM25-5, CLM26-1, CLM56-4 and CLM57-2; Table 1) were grown with shaking for 18–24 h at 30°C in NGY medium (0.1% Neopeptone, 0.4% glucose and 0.1% Yeast Extract). The *pyk1/pyk1* null mutant and its controls (CLM44-5, CLM45-9 and CLM19-3; Table 1) were grown on NGY containing 0.4% Casamino acids instead of glucose. Groups of five or six mice were inoculated via the lateral tail vein with $1.2\text{--}1.9 \times 10^4$ cfu per gram of body weight (Brand *et al.*, 2004; MacCallum and Odds, 2005). Mice were monitored over 28 days and animals showing signs of distress or illness were humanely terminated and deaths recorded as occurring the following day. The kidneys and brain were removed *post mortem*, homogenized in 0.5 ml of water, and *C. albicans* tissue burdens determined by viable counting. Viable counts were performed on glucose-containing medium for the *icl1/icl1* and *pck1/pck1* mutants, and on amino acid-containing medium for the *pyk1/pyk1* mutant. For the analysis of GFP expression *in vivo*, frozen sections of kidney tissue from infected mice were examined as described previously (Barelle *et al.*, 2004). All experimentation was carried out under the terms of the UK Home Office licenses for research on animals.

Acknowledgements

We are grateful to Zhikang Yin, David Stead, Laura Selway and Janet Walker (COGEME Proteomics Service Facility in Aberdeen) for providing protein expression data prior to publication. We thank Paul Dennison for help with the construction of the *PYK1/pyk1* mutant, and Susan Budge for excellent technical assistance. This work was funded by the Wellcome Trust (055015; 063204, 72263), the BBSRC (1/P17124, BBS/B/06679), the European Commission (MRTN-CT-2003-504148) and the University of Aberdeen.

References

Abi-Said, D., Anaissie, E., Uzun, O., Raad, I., Pinzcowski, H., and Vartivarian, S. (1997) The epidemiology of hematoge-

- nous candidiasis caused by different *Candida* species. *Clin Infect Dis* **24**: 1122–1128.
- Barelle, C.J., Manson, C., MacCallum, D., Odds, F.C., Gow, N.A.R., and Brown, A.J.P. (2004) GFP as a quantitative reporter of gene regulation in *Candida albicans*. *Yeast* **21**: 333–340.
- Brand, A., MacCallum, D.M., Brown, A.J.P., Gow, N.A.R., and Odds, F.C. (2004) Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the *RPS10* locus. *Eukaryot Cell* **3**: 900–909.
- Brown, A.J.P. (2005) Integration of metabolism with virulence in *Candida albicans*. In *Fungal Genomics*. Brown, A.J.P. (ed.). Heidelberg: Mycota XIII, Springer-Verlag, pp. 185–203.
- Calderone, R.A. (2002) *Candida and Candidiasis*. Washington DC: American Society for Microbiology Press.
- Dennison, P.M.J., Ramsdale, M., Manson, C.L., and Brown, A.J.P. (2005) Gene disruption in *Candida albicans* using a synthetic, codon-optimised Cre-*loxP* system. *Fungal Genet Biol* **42**: 737–748.
- Douglas, L.J. (2003) *Candida* biofilms and their role in infection. *Trends Microbiol* **11**: 30–36.
- Fonzi, W.A., and Irwin, M.Y. (1993) Isogenic strain construction and gene mapping in *Candida albicans*. *Genetics* **134**: 717–728.
- Fradin, C., Kretschmar, M., Nichterlein, T., Gaillardin, C., d'Enfert, C., and Hube, B. (2003) Stage-specific gene expression of *Candida albicans* in human blood. *Mol Microbiol* **47**: 1523–1543.
- Fradin, C., De Groot, P., MacCallum, D., Schaller, M., Klis, F., Odds, F.C., and Hube, B. (2005) Granulocytes govern the transcriptional response, morphology and proliferation of *Candida albicans* in human blood. *Mol Microbiol* **56**: 397–415.
- Garcia-Sanchez, S., Mavor, A., Russell, C.L., Argimon, S., Dennison, P., Enjalbert, B., and Brown, A.J.P. (2005) Global roles of Ssn6 in Tup1- and Nrg1-dependent gene regulation in the fungal pathogen, *Candida albicans*. *Mol Biol Cell* **16**: 2913–2925.
- Gillum, A.M., Tsay, E.Y., and Kirsch, D.R. (1984) Isolation of the *Candida albicans* gene for orotidine-5'-phosphate decarboxylase by complementation of *S. cerevisiae ura3* and *E. coli pyrF* mutations. *Mol Gen Genet* **198**: 179–182.
- Gow, N.A.R., and Gooday, G.W. (1982) Growth kinetics and morphology of colonies of the filamentous form of *Candida albicans*. *J Gen Microbiol* **128**: 2187–2194.
- Kaiser, C., Michaelis, S., and Mitchell, A. (1994) *Methods in Yeast Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Kullberg, B.J., and Oude Lashof, A.M. (2002) Epidemiology of opportunistic invasive mycoses. *Eur J Med Res* **7**: 183–101.
- Leuker, C.E., Sonneborn, A., Delbruck, S., and Ernst, J.F. (1997) Sequence and promoter regulation of the *PCK1* gene encoding phosphoenolpyruvate carboxykinase of the fungal pathogen *Candida albicans*. *Gene* **192**: 235–240.
- Lorenz, M.C., and Fink, G.R. (2001) The glyoxylate cycle is required for fungal virulence. *Nature* **412**: 83–86.
- Lorenz, M.C., and Fink, G.R. (2002) Life and death in a macrophage: role of the glyoxylate cycle in virulence. *Eukaryot Cell* **1**: 657–662.
- Lorenz, M.C., Bender, J.A., and Fink, G.R. (2004) Transcriptional response of *Candida albicans* upon internalization by macrophages. *Eukaryot Cell* **3**: 1076–1087.
- MacCallum, D.M., and Odds, F.C. (2005) Temporal events in the intravenous challenge model for experimental *Candida albicans* infections in female mice. *Mycoses* **48**: 151–161.
- Murad, A.M.A., Lee, P.R., Broadbent, I.D., Barelle, C.J., and Brown, A.J.P. (2000) Clp10, an efficient and convenient integrating vector for *Candida albicans*. *Yeast* **16**: 325–327.
- Murad, A.M.A., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., et al. (2001) *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. *EMBO J* **20**: 4742–4752.
- Odds, F.C. (1988) *Candida and Candidosis*, 2nd edn. London: Bailliere Tindall.
- Prigneau, O., Porta, A., Poudrier, J.A., Colonna-Romano, S., Noel, T., and Maresca, B. (2003) Genes involved in β -oxidation, energy metabolism and glyoxylate cycle are induced by *Candida albicans* during macrophage infection. *Yeast* **20**: 723–730.
- Ralph, P., Prichard, J., and Cohn, M. (1975) Reticulum cell sarcoma: an effector cell in antibody-dependent cell-mediated immunity. *J Immunol* **114**: 898–905.
- Rubin-Bejerano, I., Fraser, I., Grisafil, P., and Fink, G.R. (2003) Phagocytosis by neutrophils induces an amino acid deprivation response in *Saccharomyces cerevisiae* and *Candida albicans*. *Proc Natl Acad Sci USA* **100**: 11007–11012.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Sharkey, L.L., Liao, W.-I., Ghosh, A.K., and Fonzi, W.A. (2005) Flanking direct repeats of *hisG* alter *URA3* marker expression at the *HWP1* locus of *Candida albicans*. *Microbiology* **151**: 1061–1071.
- Sherman, F. (1991) Getting started with yeast. *Methods Enzymol* **194**: 3–21.
- Sundstrom, P., Cutler, J.E., and Staab, J.F. (2002) Reevaluation of the role of *HWP1* in systemic candidiasis by use of *Candida albicans* strains with selectable marker *URA3* targeted to the *ENO1* locus. *Infect Immun* **70**: 3281–3283.
- Velasco, E., Thuler, L.C., Martins, C.A., Nucci, M., Dias, L.M., and Goncalves, V.M. (2000) Epidemiology of bloodstream infections at a cancer centre. *Sao Paulo Med J* **118**: 131–138.
- Wilson, R.B., Davis, D., and Mitchell, A.P. (1999) Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. *J Bacteriol* **181**: 1868–1874.
- Yin, Z., Wilson, S., Hauser, N.C., Tourmu, H., Hoheisel, J.D., and Brown, A.J.P. (2003) Glucose triggers different global responses in yeast depending on the strength of the signal, and transiently stabilises ribosomal protein mRNAs. *Mol Microbiol* **48**: 713–724.
- Yin, Z., Stead, D., Selway, L., Walker, J., Riba-Garcia, I., McInerney, T., et al. (2004) Proteomic response to amino acid starvation in *Candida albicans* and *Saccharomyces cerevisiae*. *Proteomics* **4**: 2425–2436.