Amino acids mediate colony and cell differentiation in the fungal pathogen *Candida parapsilosis*

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Candida parapsilosis is responsible for severe cases of non-albicans systemic candidiasis and is one of the leading causes of mortality in neonates. The molecular mechanisms underlying this organism's virulence remain unknown. Unlike *C. albicans*, which can exist in several morphogenetic forms, *C. parapsilosis* exists in either the yeast or pseudohyphal forms. The environmental signals that trigger pseudohyphal differentiation and the signalling pathways that transduce these signals are unknown. This paper provides evidence for the role of amino acids in morphogenesis in *C. parapsilosis*. The cell and colony morphologies, pseudohyphal differentiation and invasive growth of five *C. parapsilosis* isolates were characterized in ammonium-rich minimal media lacking or supplemented with naturally occurring amino acids. *C. parapsilosis* underwent dramatic changes in cellular and colony morphology and formed pseudohyphae in response to a specific subset of amino acids. Transport studies showed that these amino acid inducers activate the transport of some, but not all, unrelated amino acids. Interestingly, citrulline, an amino acid that is not transported in the presence of ammonium, strongly induced pseudohyphal morphogenesis in *C. parapsilosis* under these conditions. Together the data suggest that amino acids are important morphogens in *C. parapsilosis* and that amino-acid-mediated morphogenesis in this organism does not require transport of the ligand across the plasma membrane.

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INTRODUCTION

Candidiasis is an important fungal disease caused by species of the genus *Candida*. Although *Candida albicans* causes the most lethal form of disease, non-albicans infections, particularly those associated with *Candida parapsilosis*, are on the rise (Fridkin *et al.*, 2006). *C. parapsilosis* is responsible for the majority of candidaemia outbreaks in neonatal and surgical intensive-care units (Bassetti *et al.*, 2006; Resende *et al.*, 2002). Most cases are device-related infections or are associated with intravenous hyperalimentation (Krcmery & Barnes, 2002). Unlike *C. albicans*, the molecular determinants of pathogenicity of *C. parapsilosis* are not yet known.

Unlike *C. albicans*, which can grow as a budding yeast, form germ tubes and differentiate into pseudohyphae and true hyphae, *C. parapsilosis* exists in either yeast or pseudohyphal forms. The transition from the yeast to the pseudohyphal form in *C. parapsilosis* has recently been shown to occur in serum medium (Laffey & Butler, 2005). However, the molecular determinants of this differentiation have not yet been identified, mostly due to the lack of genetic tools for targeted disruption. Serum is also the most effective inducer of filamentous growth in *C. albicans*, and the signal transduction pathways activated by serum have been well

characterized in this organism (for a review see Kumamoto & Vines, 2005). Early investigations in *C. albicans* suggested that the serum inducing factor(s) was heat stable and non-dialysable, and that the peak of serum induction activity co-migrated with the serum albumin fraction in gel filtration purification assays (Barlow *et al.*, 1974; Buckley & Van Uden, 1963; Reynolds & Braude, 1956). However, further studies showed that some but not all preparations of serum albumin induce germ tubes and hyphae, and even those that induce hyphal differentiation are not as potent as serum, thus arguing against serum albumin being the inducing factor (Feng *et al.*, 1999). More recent studies revealed that glucose is the major serum dialysable component responsible for hyphal differentiation in *C. albicans* (Hudson *et al.*, 2004). These studies also indicated the presence of a non-dialysable morphogenetic component, the identity of which remains to be established (Hudson *et al.*, 2004). Interestingly, several studies have suggested that, in addition to their role as building blocks in protein synthesis, amino acids might also have morphogenetic activity. Lee *et al.* (1975) found that a synthetic medium, which is commonly referred to as Lee's medium, containing a combination of various amino acids induces hyphal formation in *C. albicans*. Furthermore, Dabrowa *et al.* (1976) showed that proline can induce hyphal differentiation in *C. albicans*. The molecular mechanism by which amino acids induce hyphal differentiation has only started to be elucidated

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; DIC, differential interference contrast; RAPD, randomly amplified polymorphic DNA.

(Brega *et al.*, 2004; Martinez & Ljungdahl, 2004). Molecular studies in *C. albicans* identified Csy1, which senses amino acids in the environment and transduces signals that induce the expression of amino acid permease genes and activate amino acid uptake (Brega *et al.*, 2004). Interestingly, deletion of the *CSY1* gene resulted in severe alterations of amino acid uptake and transcription of amino acid permease genes, but also in the loss of filamentation in serum- and amino-acid-based solid media (Martinez & Ljungdahl, 2004). Furthermore, disruption of the *C. albicans* *CSH3* gene, which encodes an endoplasmic reticulum packaging chaperone important for proper targeting of amino acid permeases to the plasma membrane, results in defects in amino acid uptake, and altered colony and cell morphologies (Martinez & Ljungdahl, 2004). The link between the signalling pathways involved in the transport and utilization of amino acids and those involved in morphogenesis has not yet been established. In addition to Csy1, other membrane proteins have also been reported to play a role in amino acid sensing and morphogenesis under different conditions. Maidan *et al.* (2005a, b) in *C. albicans* and Xue *et al.* (2006) in *Cryptococcus neoformans* indicated that G-protein-coupled receptors are important for methionine-induced transition from yeast to hyphae cells. Donaton *et al.* (2003) reported that, under nitrogen starvation, the general amino acid permease Gap1 of *Saccharomyces cerevisiae* acts as an amino acid sensor for activation of protein kinase A, a pathway which also controls pseudohyphal differentiation.

Here we have investigated the role of amino acids in morphogenesis in *C. parapsilosis*. Our results suggest that amino acids are important morphogens in *C. parapsilosis* and that amino-acid-mediated morphogenesis in this organism does not require transport of the ligand across the plasma membrane.

METHODS

Strains and growth conditions. Five clinical isolates of *C. parapsilosis* (CpSH, CAp18, CAp59, CpCBM and ATCC 22019) were used. Strains were cultured in rich medium YPD (2% Bacto Peptone, 1% yeast extract, 2% glucose) or synthetic medium SD (1.7% yeast nitrogen base, 0.5% ammonium sulfate and 2% glucose). Colony morphology, cell elongation and invasion assays were performed by plating yeast cells of mid-exponential-phase cultures on either YPD containing 10% fetal bovine serum, Lee's medium (Lee *et al.*, 1975) or SD supplemented with 10 mM of individual amino acids. Cell morphology was determined by scraping the surfaces of the colonies and examining the cells by light microscopy or by growing cells in liquid media followed by microscopic analysis. For agar invasion, the plates were incubated at 37 °C for 4 days, washed with running water and then photographed.

DAPI and calcofluor staining. To stain the cell wall and nucleus of *C. parapsilosis*, 100 µl aliquots of cells grown in the indicated media were washed twice in PBS and then resuspended in 100 µl calcofluor white solution (1 mg ml⁻¹) and 10 µl DAPI solution (4',6-diamidino-2-phenylindole; 1 mg ml⁻¹). Aliquots (5 µl) of the samples were then mounted on glass slides and cells were analysed by fluorescence microscopy using a Nikon Eclipse TE2000-E microscope.

Genotype analysis. The genotyping of the five isolates of *C. parapsilosis* was performed using RAPD (randomly amplified polymorphic DNA) analysis and the primer RPO2 (5'-GCGATCCCCA-3') as previously described (Tavanti *et al.*, 2005). The *SADH* gene from the five *C. parapsilosis* isolates was PCR amplified using the primer pair GTTGATGCTGTTGGATTGT and CAATGCCAAATCTCCCAA. The resulting PCR product was digested with *Ban*I.

Amino acid transport assays. Transport assays were performed as previously described (Brega *et al.*, 2004). Overnight cultures of cells grown at 37 °C on SD medium were diluted in 20 ml SD alone or containing 10 mM of individual amino acids. Cells were harvested at OD₆₀₀ ~0.6 by centrifugation at 2000 g for 10 min at 4 °C, washed twice with cold PBS and resuspended in 700 µl SD medium. Then 100 µl of this suspension was added to 150 µl SD medium containing 0.2 µCi (7.4 kBq) ³H-labelled amino acids (Amersham Pharmacia). After incubating this mixture at 37 °C, amino acid uptake was stopped by addition of 5 ml ice-cold PBS. The cells were collected on a Whatman GF/C glass microfibre filter, washed three times with cold PBS, air-dried and their radioactivity measured in a scintillation counter (Grauslund *et al.*, 1995). All uptake studies were done in triplicate, and mean values were determined.

RESULTS

Pseudohyphal differentiation in *C. parapsilosis*

While it is well known that *C. parapsilosis* exists in both yeast and pseudohyphal forms, the mechanisms by which yeast cells differentiate into pseudohyphae are not known in this organism. To gain further insight into these processes, we

Fig. 1. Colony and cell morphology of *C. parapsilosis* CpSH in minimal and rich media. Cells were pre-grown at 37 °C in SD medium in the absence of nutritional supplements and subsequently plated on SD medium, YPD, YPD+10% serum and Lee's medium. After 4 days of incubation, colonies were visualized by light microscopy (20×) and cells from the colonies were analysed by DIC (100×).

examined the morphological changes of a *C. parapsilosis* isolate (CpSH) grown on various media known to either lack or have an effect on hyphal differentiation in other filamentous fungi. Whereas the CpSH strain formed smooth colonies with no filamentous extensions from the edge of the colonies on minimal (SD) or rich (YPD) medium lacking or supplemented with 10 % serum, it formed smooth colonies with filamentous extensions on the amino-acid-rich Lee's medium (Fig. 1). To examine whether these morphogenetic changes were directly linked to changes in cell morphology, cells were collected from the surface of the colonies and examined by differential interference contrast (DIC) (Fig. 1). Whereas on SD medium cells exhibited a typical yeast-like form, cells from colonies grown on YPD, YPD + serum or Lee's medium were elongated (Fig. 1). These data suggest that changes in colony morphology do not correlate with changes in cell morphology. We further examined the invasiveness of the strain under these four growth conditions. Strong invasive growth was detected on Lee's medium compared to SD, YPD or YPD + serum (not shown).

***C. parapsilosis* morphogenetic changes induced by single amino acids**

The changes observed on Lee's medium led us to investigate the effect of individual amino acids on the colony and cell morphologies of CpSH (Table 1). Equal amounts of cells were plated on SD medium alone or supplemented with

10 mM of one of 19 naturally occurring amino acids. On SD medium lacking amino acids all colonies were smooth, lacked filamentous extensions, contained yeast-like cells and were not invasive (Figs 2–4). Addition of some amino acids resulted in dramatic changes in colony and cell morphology. Out of the 19 amino acids tested, 9 induced major changes in the morphology of the colonies (Fig. 2). Lysine, arginine and glutamine resulted in colonies with crepe morphology; asparagine, aspartic acid and valine resulted in colonies with crater morphology; and glycine, isoleucine, threonine and tyrosine resulted in colonies with concentric morphology. Interestingly, all the amino acids induced invasive growth but none induced filamentous extensions from the edge of the colonies (Table 1) as shown for lysine, arginine and isoleucine (Fig. 3). Characterization of the morphology of the cells taken from those colonies revealed that, out of the 19 amino acids tested, only arginine, aspartic acid, glutamine, histidine, leucine, lysine, phenylalanine and proline led to the formation of elongated cells (Fig. 4). Cell elongation was further characterized on liquid media by inoculating yeast cells, preincubated in SD medium lacking amino acids, into SD medium alone or supplemented with single amino acids. Results similar to those obtained with solid media were found (Table 1). The nuclear content of *C. parapsilosis* cells grown in SD medium lacking amino acids or supplemented with either arginine or lysine, which induced cell elongation, or isoleucine, which did not affect

Table 1. Amino-acid-mediated morphogenesis in five *C. parapsilosis* isolates

C, colony morphology; I, invasive growth; CE, cell elongation in liquid medium; EF, filamentation from the edge of the colony; SM, smooth; SN, snowball; CP, crepe; CT, crater; CO, concentric; Y, yeast; E, elongated.

	CpSH				CAp59				CAp18				ATCC 22109				CpCBM			
	C	I	CE	EF	C	I	CE	EF	C	I	CE	EF	C	I	CE	EF	C	I	CE	EF
SD	SM	–	Y	–	SM	–	Y	–	SM	–	Y	–	SM	–	Y	–	SM	–	Y	–
SD + Ala	SM	+	Y	–	SM	–	Y	–	SM	–	Y	–	CO	+	Y	–	SM	+	Y	–
SD + Arg	CP	+	E	–	SM	+	E	+	SM	+	E	+	SM	+	E	+	SM	+	E	–
SD + Asn	CT	+	Y	–	SM	+	Y	–	SM	+	Y	–	SM	+	Y	–	SM	+	Y	–
SD + Asp	CT	+	E	–	SM	+	E	–	SN	+	E	–	SN	+	E	–	SM	+	Y	–
SD + Gln	CP	+	E	–	SM	+	Y	–	SM	+	E	–	CO	+	E	–	SM	+	Y	–
SD + Glu	SM	+	Y	–	SM	+	Y	–	SM	+	Y	–	CO	+	Y	–	SM	+	Y	–
SD + Gly	CO	+	Y	–	CO	+	Y	–	SM	+	Y	–	SM	+	Y	–	SM	+	Y	–
SD + His	SM	+	E	–	CO	+	Y	–	CO	+	E	–	CO	+	E	–	SM	+	E	–
SD + Ile	CO	+	Y	–	SM	+	Y	–	SM	+	Y	–	SM	+	Y	–	SM	+	Y	–
SD + Leu	SM	+	E	–	SM	–	Y	–	SN	–	Y	–	SN	–	E	–	SM	+	Y	–
SD + Lys	CP	+	E	–	CO	+	E	–	CO	+	E	–	CO	+	E	–	SM	+	E	–
SD + Met	SM	+	Y	–	SM	–	Y	–	SN	–	Y	–	SM	–	Y	–	SM	–	Y	–
SD + Phe	SM	+	E	–	SM	–	Y	–	CP	–	Y	–	SM	+	Y	–	SM	+	Y	–
SD + Pro	SM	+	E	–	CO	–	Y	–	SM	–	Y	–	SM	–	E	–	SM	+	E	–
SD + Ser	SM	+	Y	–	SM	–	Y	–	SM	–	E	–	SM	+	Y	–	SM	+	Y	–
SD + Thr	CO	+	Y	–	SM	–	Y	–	SM	–	Y	–	CO	+	Y	–	SM	+	Y	–
SD + Trp	SM	+	Y	–	CO	–	Y	–	CO	–	Y	–	CO	–	E	–	SM	+	Y	–
SD + Tyr	CO	+	Y	–	CO	–	Y	–	CO	–	Y	–	CO	+	Y	–	SM	+	Y	–
SD + Val	CT	+	Y	–	SM	–	Y	–	SM	–	Y	–	SM	–	Y	–	SM	+	Y	–

Fig. 2. Morphology of *C. parapsilosis* CpSH colonies on SD medium supplemented with single amino acids. Cells were pre-grown at 37 °C in SD medium in the absence of nutritional supplements and subsequently plated on SD medium supplemented with 10 mM of each of the 19 amino acids indicated. After 4 days of incubation, colonies were visualized by light microscopy. A, alanine; R, arginine; N, asparagine; D, aspartic acid; Q, glutamine; E, glutamic acid; G, glycine; H, histidine; I, isoleucine; L, leucine; K, lysine; M, methionine; F, phenylalanine; P, proline; S, serine; T, threonine; W, tryptophan; Y, tyrosine; V, valine.

morphogenesis, was further examined by DAPI staining. As shown in Fig. 5, arginine and lysine induced the formation of a long chain of elongated cells each containing a single nucleus and separated by a septum, as revealed by calcofluor, which stains the cell wall; whereas in the absence of amino acids or in the presence of isoleucine only yeast cells containing a single nucleus per cell and attached to each other were detected (Fig. 5). These data thus indicate that amino-acid-mediated cell elongation is not a result of an abnormal cell separation.

Regulation of amino acid transport by external amino acids in *C. parapsilosis*

In different yeast species, amino acid transport involves specific and general amino acid permeases, and is a co-ordinated process that involves a plasma membrane sensor that detects the presence of external amino acids and transduces signals to activate the expression of AAP genes (Brega *et al.*, 2004; Didion *et al.*, 1998; Iraqui *et al.*, 1999; Klasson *et al.*, 1999). To understand the mechanism by which amino acids induce morphogenesis in *C. parapsilosis*, we examined whether amino-acid-mediated morphogenesis and amino acid transport are linked. Amino acid transport was determined by measuring the rate of uptake of two

Fig. 3. Invasive growth and migration of *C. parapsilosis* CpSH on SD medium supplemented with single amino acids. (A) Cells were pre-grown at 37 °C in SD medium in the absence of nutritional supplements and subsequently plated on SD medium supplemented with 10 mM of each of the amino acids indicated (R, arginine; K, lysine; I, isoleucine). After 4 days of incubation, the plates were washed with running water and visualized by light microscopy (20 $\times$). (B) The edge of the CpSH colonies grown at 37 °C in SD medium lacking or supplemented with 10 mM of lysine, arginine or isoleucine was examined by light microscopy (20 $\times$).

radiolabelled amino acids, valine and isoleucine, in CpSH cells grown in SD medium alone or preincubated with lysine for 3 h (during which all cells remained in the yeast form). At 37 °C the transport of both substrates was linear during the first 10 min before reaching a plateau (Fig. 6A, B), and no transport could be measured at 0 °C. These data suggest that the uptake of amino acids in *C. parapsilosis* is transporter-mediated. Interestingly, a significantly higher uptake of valine and isoleucine could be measured in cells incubated in SD medium supplemented with lysine compared to those incubated in SD medium lacking amino acids (Fig. 6A, B). We further examined the effect of lysine and arginine on the transport of various other substrates. The transport of glutamine, phenylalanine, proline, tyrosine and leucine was three- to sevenfold higher in the presence of

Fig. 4. Morphology of *C. parapsilosis* CpSH cells on SD medium supplemented with single amino acids. Cells were pre-grown at 37 °C in SD medium in the absence of nutritional supplements and subsequently plated on SD medium supplemented with 10 mM of each of the 19 amino acids listed (see Fig. 2 for key to abbreviations). After 4 days of incubation, cells from the colonies were analysed by DIC (100×).

lysine or arginine than in the absence of amino acids (Fig. 6C). To assess whether the activation of amino acid transport by specific ligands correlates with the ability of those ligands to induce morphogenesis, the transport of radiolabelled valine was measured in SD medium alone or supplemented with amino acids that either induce cell elongation or lack this morphogenic property (Fig. 6D).

Independent of their ability to induce cell elongation, all the amino acids resulted in an increase in valine uptake. The level of induction, however, varied depending on the ligand used, with the highest induction obtained with histidine (12-fold) and glutamine (9-fold) and the lowest induction obtained with alanine (3-fold), phenylalanine (2.5-fold) and threonine (2-fold). These data suggest that amino-acid-mediated activation of morphogenesis and amino-acid-mediated activation of amino acid transport are not coupled.

Amino-acid-mediated morphogenesis does not require entry of amino acids into the cell

The morphogenetic and transport analyses suggested a complex mechanism by which external amino acids can induce either cell differentiation and/or amino acid transport. To assess whether amino-acid-mediated morphogenesis requires entry of the inducer into the cell, we examined the transport of citrulline, which in other species is known to require a general amino acid permease and is controlled by the quality of the available nitrogen source (Jauniaux & Grenson, 1990; Soetens *et al.*, 2001; Stanbrough & Magasanik, 1995; ter Schure *et al.*, 1995). Therefore, the time-dependent uptake of citrulline was measured in the presence of preferred (ammonia) or non-preferred (proline or urea) nitrogen sources. No uptake of citrulline could be detected in ammonium medium, whereas a saturable uptake could be measured in media containing proline or urea as sole nitrogen sources (Fig. 7A). Interestingly, incubation of CpSH cells with citrulline in SD plates containing ammonia resulted in cell and colony morphologies similar to those obtained with lysine and arginine (Fig. 7B). We further monitored the time-dependent differentiation of CpSH in response to citrulline in liquid medium containing ammonium. As shown in Fig. 8, under these conditions, citrulline resulted in the formation of very long chains of elongated cells, each containing a single nucleus

Fig. 5. Time-dependent cell elongation of *C. parapsilosis* CpSH. Cells were preincubated in SD medium overnight and then subcultured into liquid SD medium lacking or supplemented with 10 mM arginine (R), lysine (K) or isoleucine (I). Samples were collected at 0 and 24 h after incubation at 37 °C and visualized by DIC. DNA was counterstained with DAPI (blue) (A) and cell wall was stained with calcofluor white (CFW, green) (B) as described in Methods.

Fig. 6. Amino-acid-mediated activation of amino acid transport in *C. parapsilosis* CpSH. (A, B) Time-dependent uptake of radiolabelled valine (A) or isoleucine (B) in SD medium lacking (□) or supplemented with (■) 10 mM lysine. (C) Transport of valine (Val), glutamine (Gln), phenylalanine (Phe), proline (Pro), tyrosine (Tyr), leucine (Leu) and lysine (Lys) in unsupplemented SD medium (white bars), or SD medium supplemented with 10 mM lysine (grey bars) or arginine (black bars). (D) Effects of various amino acids on the transport of radiolabelled valine. Cells were grown in the absence of the inducers, transferred to medium supplemented with 10 mM of the inducer and incubated at 37 °C for 3 h. After cell wash, transport analyses were performed for 4 min (initial rate of uptake) at 37 °C as described in Methods. Uptake values were normalized to those of CpSH in SD medium.

and separated by a septum. As a control, no changes in cell morphology could be detected in SD medium lacking amino acids (Fig. 8). Together these data demonstrate that citrulline-mediated morphogenesis is independent of the ability of this amino acid to enter the cell.

Amino-acid-mediated morphogenesis in different *C. parapsilosis* isolates

To examine whether the effects seen with external amino acids on the morphogenetic differentiation of the CpSH strain were general, similar assays were performed in different clinical isolates of *C. parapsilosis*. Four clones of *C. parapsilosis*, CpCBM, ATCC 22019, CAP59 and CAP18, were subcloned and authenticated as *C. parapsilosis* isolates using the RapID Yeast Plus System test. Recent studies have indicated that *C. parapsilosis* isolates can be divided into three different groups based on their RAPD patterns as well as by analysis of the digestion pattern of a PCR-amplified *SADH* gene encoding secondary alcohol dehydrogenase (Tavanti *et al.*, 2005). RAPD analysis using genomic DNA from CpCBM, ATCC 22019, CAP59, CAP18 and CpSH revealed a pattern similar to that of *C. parapsilosis* clones of group I (not shown) (Tavanti *et al.*, 2005). Similarly, amplification of the *SADH* gene identified a PCR fragment

of 716 bp (not shown) that upon digestion with *BanI* gave rise to two fragments, of 521 bp and 196 bp (not shown). This restriction pattern is specific for clones of group I (Tavanti *et al.*, 2005). The colony morphology, cell elongation and invasive growth of the five strains were analysed in solid SD medium lacking or supplemented with individual amino acids (Table 1) as well as on the amino-acid-based Lee's medium. With the exception of serine, which did not alter the colony morphology of any of the five *C. parapsilosis* isolates analysed in this study, the remaining 18 amino acids all induced changes in colony morphology in isolates CAP59, CAP18 and ATCC 22019. However, the type of colony morphology and the amino acids responsible for these changes were different from those observed in CpSH. Thus, the seven amino acids that had no effects on colony morphology of CpSH resulted in colonies of snowball or concentric shapes in the three other isolates. Conversely, arginine and glutamine, which induced crepe morphology in CpSH, resulted in colonies of smooth morphology in CAP59, CAP18 and ATCC 22019. On the other hand, lysine, which induced crepe morphology in CpSH, resulted in colonies of concentric morphology in CAP59, CAP18 and ATCC 22019. Surprisingly, no changes in the colony morphology of the CpCBM isolate could be detected on amino-acid-containing plates. Analysis of the cell morphology showed

Fig. 7. Citrulline uptake and morphogenetic effects in *C. parapsilosis* CpSH. (A) Cells were precultured at 37 °C in minimal medium containing 38 mM ammonium sulfate (SD), washed extensively with water then diluted to 1×10^4 cells ml^{-1} in minimal medium containing ammonium sulfate (SD), minimal medium containing ammonium sulfate and 10 mM citrulline (SDC), or minimal medium lacking ammonium but supplemented with 10 mM urea (SU) or 10 mM proline (SP). Cells were incubated at 37 °C for 6 h, after which they were harvested and used to measure the transport of citrulline as described in Methods. (B) Cells were precultured at 37 °C in SD medium and then plated on plates of SD medium lacking or supplemented with 10 mM citrulline (SDC) and incubated at 37 °C for 4 days. The effect of citrulline on cell elongation was determined by liquid culture after incubation of CpSH cells in SD medium lacking or supplemented with 10 mM citrulline (SDC) for 6 h at 37 °C. Cells were analysed by DIC.

that of the 19 amino acids analysed, alanine, asparagine, glutamine acid, glycine, isoleucine, methionine, threonine, tyrosine and valine had no effect on cell elongation; arginine and lysine induced cell elongation in all five isolates; and the ability of the remaining eight amino acids to induce cell elongation varied depending on the isolate. Analysis of the amino-acid-mediated invasiveness phenotype showed that arginine, asparagine, aspartic acid, glutamine, glutamic acid, glycine, histidine, isoleucine and lysine induced invasive growth in all five isolates (Table 1, Fig. 9C). Leucine and methionine, which induced invasive growth in CpSH, failed to trigger this phenotype in CAP59, CAP18, ATCC 22019 and CpCBM. The remaining eight amino acids varied in their ability to induce invasive growth depending on the isolate tested (Table 1). Interestingly, of the 19 amino acids

tested, only arginine induced the formation of filaments that extended from the edge of the colonies in isolates CAP59, CAP18 and ATCC 22019 (Fig. 9D). On Lee's medium, all isolates formed extensions from the edge of the colonies and invaded the agar (Fig. 9C, D). However on this medium, only CAP18 and ATCC 22019 formed wrinkled colonies (Fig. 9B). Addition of lysine, arginine or isoleucine to Lee's medium had little or no effect on filament formation from the edge of the colonies (not shown). Interestingly, whereas only cells from within the ATCC 22019 and CpSH colonies were elongated (Fig. 9A), cells collected from the filament extensions of the five colonies displayed an elongated morphology (not shown).

DISCUSSION

In this study, we have investigated the effect of amino acids on the yeast-to-pseudohyphae differentiation, invasiveness, filamentation from the edge of the colony and change in colony morphology in the fungal pathogen *C. parapsilosis*. Our experiments using five *C. parapsilosis* isolates showed that all the amino acids tested induced at least one of the four morphogenetic changes characterized in this study. With the exception of serine, which did not alter the colony morphology of any of the five *C. parapsilosis* isolates, all amino acids induced changes in colony morphology in three other isolates in addition to CpSH, the isolate used in most of the experiments: CAP59, CAP18 and ATCC 22019. However, the type of colony morphologies and the amino acids responsible for these changes were different from those observed in CpSH. Independent of their effect on colony morphology, all the amino acids resulted in increased invasive growth on agar plates. The invasiveness, however, did not correlate with changes in cellular morphology, for out of the 19 amino acids tested only arginine, aspartic acid, glutamine, histidine, leucine, lysine, phenylalanine and proline led to the formation of elongated cells in all *C. parapsilosis* isolates tested (Fig. 4). Surprisingly, one isolate, CpCBM, showed no significant changes in colony morphology in response to amino acids, although at the cellular level, CpCBM cells underwent yeast-to-pseudohyphae differentiation in the presence of arginine, histidine, lysine and proline. At this stage the molecular mechanism by which amino acids trigger changes in colony morphology, invasiveness and cell elongation, and the molecular bases for the differences observed between the various *C. parapsilosis* isolates, remain unknown.

To gain further insight into the mechanism by which amino acids induce morphogenesis in *C. parapsilosis*, we examined whether amino-acid-mediated morphogenesis and amino acid transport in isolate CpSH were coupled. Biochemical studies revealed that amino acid entry in this strain was transporter-mediated, and was strongly induced by lysine and arginine. Interestingly, independent of their ability to induce cell elongation, invasive growth or a change in colony morphology, all the amino acids tested resulted in an increase in the uptake of radiolabelled valine, but the level of

Fig. 8. Time-dependent citrulline-mediated cell elongation of *C. parapsilosis* CpSH. (A) Cells were preincubated in SD medium overnight and then subcultured into liquid SD medium lacking or supplemented with 10 mM citrulline (SDC). Samples were collected at 0, 6, 24 and 48 h after incubation at 37 °C and were visualized by DIC (100 ×). DNA was counterstained with DAPI (blue). (B) Cells were preincubated in SD medium overnight and then subcultured into liquid SD medium lacking or supplemented with 10 mM citrulline (SDC). Samples were collected at 0, 6, 24 and 48 h after incubation at 37 °C and visualized by DIC (100 ×). Cell wall was stained with calcofluor white (CFW, green) as described in Methods.

Fig. 9. Colony and cell morphology and invasive growth of *C. parapsilosis* isolates. The five isolates were pre-grown at 37 °C in SD medium in the absence of nutritional supplements and subsequently plated on SD medium lacking or supplemented with arginine (R) or lysine (K) or on Lee's medium. After 4 days of incubation at 37 °C, the isolates were analysed for cell morphology (A), colony morphology (B), invasive growth (C) and filament extension from the edge of the colonies (D).

induction varied depending on the ligand used. For those amino acids that triggered both morphogenetic changes and increased uptake of amino acids, it was necessary to determine whether amino acid entry was required for the inducer to trigger morphogenesis. Therefore, we searched for conditions that could allow uncoupling of amino acid transport and pseudohyphal differentiation. Our studies showed that the amino acid citrulline was not transported by *C. parapsilosis* in ammonium medium, but, as in other yeast species, its transport occurred in the presence of non-preferred nitrogen sources such as proline or urea. Interestingly, incubation of CpSH cells with citrulline caused a time-dependent cell elongation and resulted in colonies with crepe morphology under conditions where citrulline was not transported (i.e. ammonium medium). These results indicate that citrulline is capable of triggering a morphogenetic change without being transported inside the cell, and further demonstrate that amino-acid-mediated morphogenesis and transport are not coupled. Together these findings point to a complex process by which amino acids trigger morphogenesis in *C. parapsilosis*. We hypothesize that amino acids are recognized as morphogenetic substrates by one or multiple membrane receptors whose stimulation by external amino acids results in the activation of specialized signal transduction pathways that control colony morphology, cell shape or invasiveness. The ongoing sequencing project of *C. parapsilosis* has revealed the presence of two putative membrane proteins that we refer to as CpSsy1 and CpGpr1 that are highly identical to *C. albicans* Csy1 and Gpr1. Current efforts to develop new tools for genetic manipulation of this organism (Nosek *et al.*, 2002) will set the stage for advanced molecular studies to characterize the role of these proteins in amino-acid-mediated morphogenesis in *C. parapsilosis*, and will help identify the components of the signal transduction pathways that control morphogenesis in this organism.

Relevant to the current study, most outbreaks of candidemia in neonatal intensive care units around the world are associated with hyperalimentation (Chowdhary *et al.*, 2003; Kataoka *et al.*, 1995; Lee *et al.*, 1998; Leibovitz *et al.*, 1992; Levy *et al.*, 1998; MacDonald *et al.*, 1998; Sherertz *et al.*, 1992; Shian *et al.*, 1993; Shin *et al.*, 2002). Interestingly, the hyperalimentation regimen contains amino acids at concentrations far higher than those required to induce hyphal differentiation. Although a direct correlation between morphogenesis and virulence has not yet been established for *C. parapsilosis*, our current findings could help establish better strategies for management of *Candida* infections, for example by monitoring the concentration of essential and nonessential amino acids administered in the hyperalimentation regimen or by finding alternative approaches to offset nitrogen loss or to treat negative nitrogen balance.

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