



# Role of the Inducible Adhesin CpAls7 in Binding of *Candida parapsilosis* to the Extracellular Matrix under Fluid Shear

Matthew N. Neale,<sup>a,b</sup> Kyle A. Glass,<sup>a,b\*</sup> Sarah J. Longley,<sup>a,b</sup> Denny J. Kim,<sup>a,b</sup> Sonia S. Laforce-Nesbitt,<sup>a,b</sup> Jeremy D. Wortzel,<sup>a,b\*</sup>  
 Sunil K. Shaw,<sup>a,b</sup>  Joseph M. Bliss<sup>a,b</sup>

<sup>a</sup>Department of Pediatrics, Women & Infants Hospital of Rhode Island, Providence, Rhode Island, USA

<sup>b</sup>Warren Alpert Medical School of Brown University, Providence, Rhode Island, USA

**ABSTRACT** The yeast *Candida parapsilosis* is an increasingly common cause of systemic fungal infections among immunocompromised individuals, including premature infants. Adhesion to host surfaces is an important step in pathogenesis, but this process has not been extensively studied in this organism. A microfluidics assay was developed to test the ability of *C. parapsilosis* to adhere to immobilized host extracellular matrix proteins under physiological fluid shear conditions. Growth in mammalian tissue culture medium at 37°C for 3 to 6 h led to the induction of an adhesive phenotype at shear forces of 1 to 5 dynes/cm<sup>2</sup> in some isolates of *C. parapsilosis*. Glutamic acid, proline, and calcium appeared to be the minimally necessary requirements for increased adhesion in these assays. To determine whether genes homologous to the ALS gene family of *C. albicans* were important for the adhesive phenotype, the expression levels of 5 homologous *C. parapsilosis* genes were quantified by using quantitative PCR (qPCR) under conditions leading to increased adhesion. *CPAR2\_404800* (*CpALS7*) and *CPAR2\_404780* showed increased expression levels compared to those in control yeast. The extent of adhesion was variable among different isolates, and linear regression identified the expression of *CpALS7* but not *CPAR2\_404780* as having a strong positive correlation with adhesion. A homozygous *CpALS7* deletion strain was deficient in adhesion, whereas the expression of *CpALS7* in *Saccharomyces cerevisiae* resulted in increased adhesion. Together, these data provide strong evidence that *CpAls7* aids in the adherence of *C. parapsilosis* to the extracellular matrix under shear forces and support its previously reported role in virulence.

**KEYWORDS** adhesion, *Candida parapsilosis*, shear force, extracellular matrix

*Candida* species are the leading cause of systemic, invasive fungal infections worldwide (1). Occurring primarily among immunocompromised individuals, these infections are initiated by a compromise of mucosal or skin barriers, access to the bloodstream, and subsequent escape from the blood to invade deeper tissues (1, 2). Given this sequence, mechanisms by which *Candida* can interact with and then breach blood vessel walls are likely important components of pathogenesis.

*Candida albicans* causes the majority of invasive infections (3) and is widely considered to be the most virulent species. The shift in morphology from its budding yeast form to a filamentous, hyphal form is well recognized as a key virulence determinant (4). Adhesion of *C. albicans* to host surfaces has been extensively studied. Although initial contact with epithelial and endothelial cells is likely made by the yeast form, initiation of hypha formation follows rapidly, and novel adhesins that have key roles in further adhesion are expressed on the hyphal surface (5).

The best-studied family of adhesins in *C. albicans* is the ALS (agglutinin-like sequence) gene family (6). This family is comprised of at least eight genes that encode large glycosylphosphatidylinositol (GPI)-linked glycoproteins on the cell surface (7).

Received 4 December 2017 Returned for  
modification 19 December 2017 Accepted  
20 January 2018

Accepted manuscript posted online 29  
January 2018

**Citation** Neale MN, Glass KA, Longley SJ, Kim DJ, Laforce-Nesbitt SS, Wortzel JD, Shaw SK, Bliss JM. 2018. Role of the inducible adhesin *CpAls7* in binding of *Candida parapsilosis* to the extracellular matrix under fluid shear. *Infect Immun* 86:e00892-17. <https://doi.org/10.1128/IAI.00892-17>.

**Editor** George S. Deepe, University of Cincinnati

**Copyright** © 2018 American Society for Microbiology. All Rights Reserved.

Address correspondence to Joseph M. Bliss, [jbliss@wihri.org](mailto:jbliss@wihri.org).

\* Present address: Kyle A. Glass, Boston University, Boston, Massachusetts, USA; Jeremy D. Wortzel, University of Pennsylvania, Philadelphia, Pennsylvania, USA.

S.K.S. and J.M.B. contributed equally.

Several *ALS* genes have been shown to confer adhesion to mammalian host surfaces (8). Of note, *ALS3* has been shown to mediate the binding of *C. albicans* to a variety of biotic matrices (6). It has also been implicated in binding to N-cadherin to initiate endocytosis by endothelial cells (9) and may also have a role in the penetration of the central nervous system (10). The induction of *ALS3* occurs with hyphal growth (11), and its expression has been detected in yeast isolated from a murine model of disseminated candidiasis and from patients with oropharyngeal candidiasis (12).

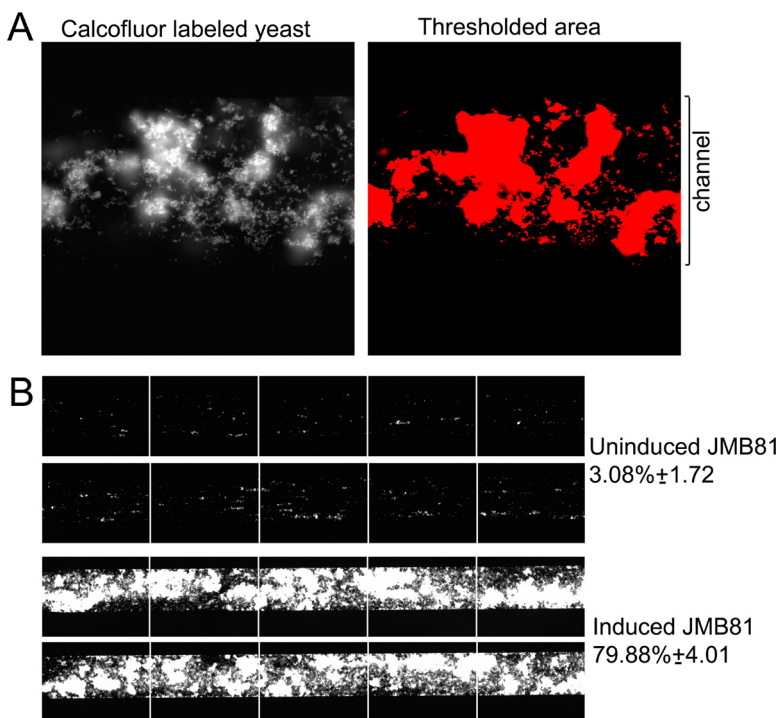
Although the majority of cases of nosocomial candidiasis is attributed to *C. albicans*, the emergence of non-*albicans* *Candida* species has been a growing cause for concern (13, 14). In particular, *Candida parapsilosis* has been documented as the non-*albicans* *Candida* species most commonly isolated from blood cultures in multiple studies; in some populations, it has surpassed *C. albicans* as the most frequently isolated *Candida* species (15). Despite the increasing recognition of *C. parapsilosis* as a clinically significant opportunistic pathogen, it remains poorly characterized relative to *C. albicans* (16). Thus, there is a need to further investigate the pathobiology of *C. parapsilosis*, with an eye toward investigating virulence mechanisms, identifying potential drug targets, and developing novel therapeutics to better combat candidiasis caused by non-*albicans* *Candida* species.

Recent efforts have shed light on putative adhesins in the *C. parapsilosis* genome. Proteomic analysis of a cell wall extract from *C. parapsilosis* identified multiple proteins that bound to fibronectin, vitronectin, and/or laminin (17). Among these proteins were those encoded by *CPAR2\_404800* and *CPAR2\_404780*, which bear significant identity to the *ALS* gene family (17). These same 2 gene products were purified from extracts of *C. parapsilosis* surface proteins based on their binding affinity for human plasminogen and high-molecular-mass kininogen (18). Furthermore, the site-specific deletion of *CPAR2\_404800* reduced the adhesion of *C. parapsilosis* to buccal epithelial cells and significantly attenuated virulence in a murine model of urinary tract infection (19). This gene was selected for targeting in this study because its product bears the highest sequence identity to *C. albicans* Als3. The authors of that study named this gene *CpALS7* based on synteny with *C. albicans* *ALS7* according to the *Candida* Gene Order database (<http://cgob3.ucd.ie/>) (20, 21). For consistency, this gene is referred to as *CpALS7* throughout this study. These recent findings suggest that *ALS* homologues in *C. parapsilosis* carry an adhesive function and may be important players in the pathogenesis of invasive infection.

The objective of this study was to identify conditions that induce adhesion in *C. parapsilosis* and examine differential *CpALS* gene expression under adhesive and non-adhesive conditions, with the goal of further characterizing adhesins in *C. parapsilosis*. In the bloodstream, *Candida* spp. encounter fluid shear forces. In order to establish deep-seated infections, *Candida* in the bloodstream must adhere to vessel walls while resisting hemodynamic shear forces. To better model this milieu *in vitro*, we measured the adhesion of *C. parapsilosis* to immobilized substrates under physiological fluid shear using a microfluidics apparatus.

## RESULTS

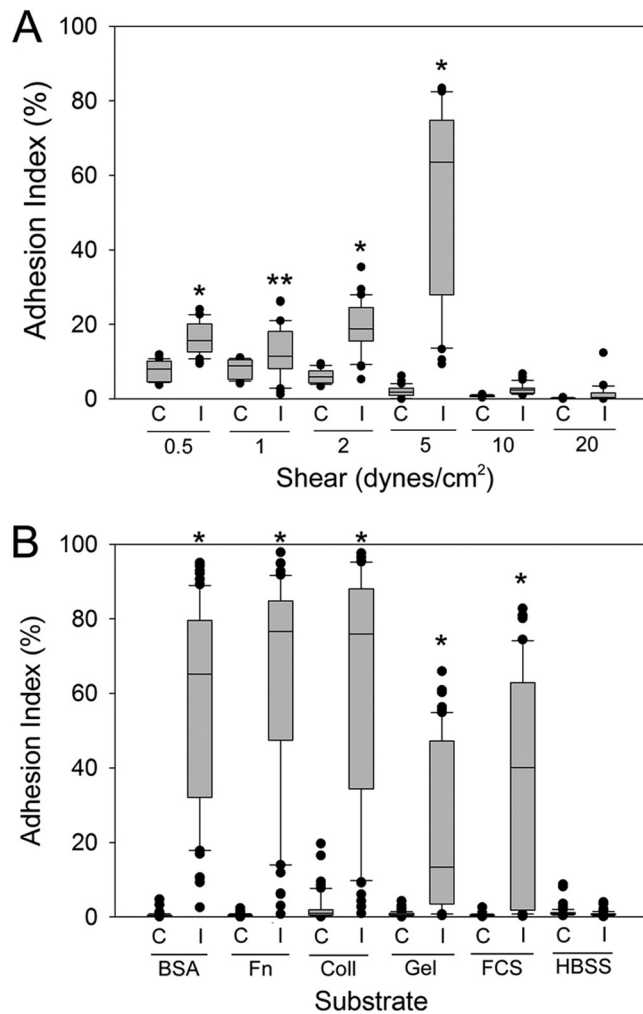
**Effect of growth conditions on adhesion of *C. parapsilosis* to host proteins under shear.** The induction of hyphal growth in *C. albicans* leads to the expression of novel proteins that have a role in adhesion to host structures. To test whether similar growth conditions would increase the adhesion of *C. parapsilosis*, strain JMB81 was grown in mammalian tissue culture medium 199 (M199) for 3 h at 37°C. Of note, in this and all subsequent experiments, the morphology of all of the strains studied under any growth condition was uniformly the yeast form. No elongated yeast cells or pseudo-hyphae were observed under any conditions. The capacity of *C. parapsilosis* yeast cells "induced" under these conditions to adhere to immobilized bovine serum albumin (BSA) was tested in a microfluidics adhesion assay under various shear forces. *C. parapsilosis* cells induced in M199 showed greatly increased adhesion under fluid shear at 5 dynes/cm<sup>2</sup> compared to control yeast cells (Fig. 1B and 2A). Under each shear force



**FIG 1** *C. parapsilosis* adhesion under fluid shear. (A, left) Calcofluor-labeled fluorescent yeast cells were observed to adhere to the microchannel under specific conditions. (Right) Fluorescence-thresholded area. (B) Microchannels under each condition were run in duplicate and are represented as a series of adjacent images encompassing approximately 80% of the channel area. The top pair shows control *C. parapsilosis* cells adhering to immobilized BSA under fluid shear at 5 dynes/cm<sup>2</sup>. The bottom pair shows induced yeast cells showing a consistently increased adhesion index under these conditions. The fluid flow direction is from right to left. The adhesion index represents the area of the channel covered by yeast, expressed as a percentage  $\pm$  the standard deviation, as indicated.

tested, the adhesion of induced yeast cells was significantly higher than that of control yeast cells, with peak adhesion at 5 dynes/cm<sup>2</sup> (Fig. 2A). Furthermore, increases in shear from 1 to 2 and from 2 to 5 dynes/cm<sup>2</sup> resulted in significant increases in the adhesion of induced yeast cells ( $P < 0.001$  in each case). In contrast, control yeast cells showed a significant decrease in adhesion with each increase in shear force above 1 dyne/cm<sup>2</sup>, as expected ( $P < 0.001$ ). Similar results were obtained with four distinct clinical isolates of *C. parapsilosis* (22, 23), where induction resulted in greater adhesion at 5 dynes/cm<sup>2</sup> than that at 1 dyne/cm<sup>2</sup> (not shown). Adhesion to different substrates was compared between control and induced *C. parapsilosis* cells. Induction increased adhesion significantly toward BSA, fibronectin, collagen, and gelatin (Fig. 2B). In contrast, channels left uncoated with any substrate (Hanks' balanced salt solution [HBSS]) showed very low adhesion for both control and induced yeast cells.

To further define the components of M199 that may have led to increased adhesion, assays were conducted following induction in a variety of media. Parallel incubation of *C. parapsilosis* in yeast extract-peptone-dextrose (YPD) medium, water, or DPBS<sup>+</sup> (Dulbecco's phosphate-buffered saline containing Ca<sup>2+</sup> and Mg<sup>2+</sup>) did not result in increased adhesion; similarly, control yeast cells (grown in YPD medium) resuspended in M199 immediately prior to loading into the assay mixture did not show increased adhesion (not shown). Incubation in HBSS<sup>+</sup> (HBSS containing calcium, magnesium, and 10 mM HEPES) also did not promote adhesion, so various components present in M199 but absent in HBSS<sup>+</sup> were added individually or in combinations to HBSS<sup>+</sup> during induction (Fig. 3). Small but significant increases in adhesion were observed when aspartic acid (D), glutamic acid (E), and proline (P) were added in combination to HBSS<sup>+</sup>. Assessment of each amino acid individually suggested that either glutamic acid or proline, but not aspartic acid, was sufficient to induce adhesion. Higher concentra-

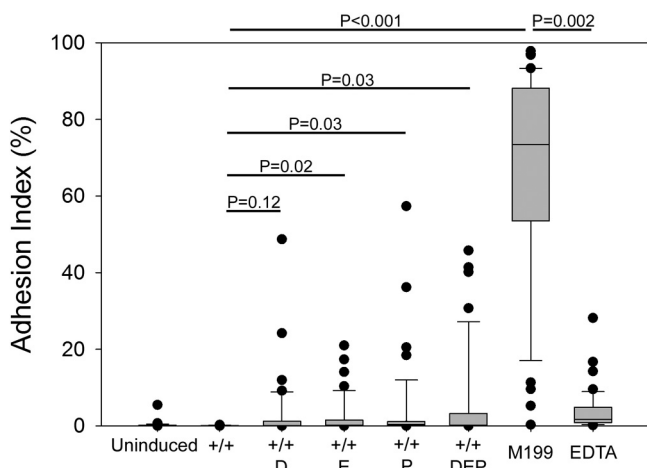


**FIG 2** Effect of shear force and the substrate on *C. parapsilosis* adhesion. *C. parapsilosis* was induced by growth in M199 for 3 h at 37°C. (A) Adhesion assays were conducted in a microfluidics environment under various shear forces. The adhesion index was calculated from duplicate channels with a minimum of 3 biological replicates. Induced yeast cells (I) were compared to control yeast cells (C) at each shear force via a Mann-Whitney rank sum test. \*,  $P < 0.001$ ; \*\*,  $P = 0.02$  (for induced versus control yeast cells at each shear force). (B) Adhesion assays were conducted as described above, at 5 dynes/cm<sup>2</sup>, over microfluidics channels coated with the indicated substrates (BSA, bovine serum albumin; Fn, fibronectin; Coll, collagen; Gel, gelatin; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution plus Ca<sup>2+</sup>/Mg<sup>+</sup>). \*,  $P < 0.001$ .

tions of these amino acids were tested and failed to further augment adhesion (not shown). Furthermore, the addition of EDTA to M199 after induction substantially reduced adhesion compared to that with M199 alone, suggesting that divalent cations are also required for yeast adhesion under flow.

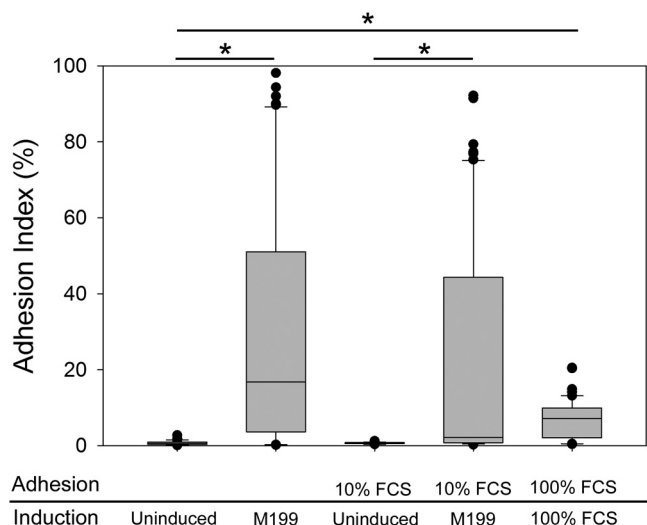
Induction in M199 medium resulted in increased adhesion to immobilized BSA, even when 10% fetal bovine serum was added during adhesion under shear (Fig. 4). Induction of yeast cells in 100% serum followed by shear flow in 100% serum also resulted in significantly increased adhesion compared to that of control yeast cells.

**Positive correlation of *CpALS7* expression with adhesion.** To identify genes that may have a role in the increased adhesion of *C. parapsilosis* grown in M199, homologues of the *ALS* gene family of *C. albicans* (8, 24) were identified by using the *Candida* Genome Database (16). Five candidate genes were identified (see Table 2), and primers appropriate for quantitative PCR (qPCR) analysis of each gene transcript were designed and validated. Only 2 genes, *CpALS7* and *CPAR2\_404780*, demonstrated a significant increase in expression in M199 relative to that in YPD medium (Fig. 5). Among the *ALS*

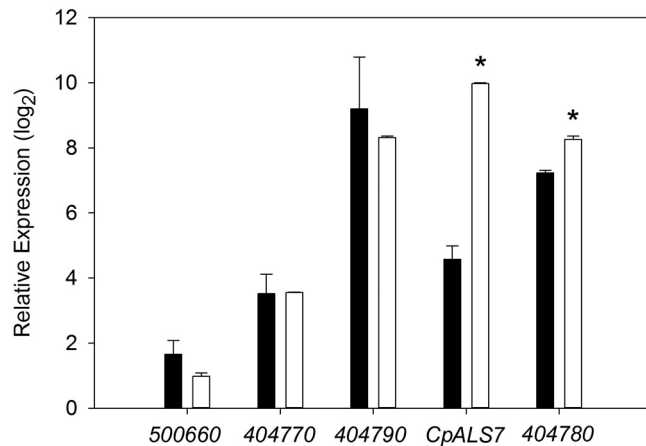


**FIG 3** Components of M199 that contribute to adhesion. *C. parapsilosis* strain JMB81 was induced by growth in the indicated media for 3 h at 37°C and tested in adhesion assays. +/+, HBSS<sup>+</sup>; D, aspartic acid; E, glutamic acid; P, proline. The adhesion index was calculated from duplicate channels with a minimum of 3 biological replicates. Statistical analysis was performed by using Kruskal-Wallis analysis of variance on ranks, and between-group comparisons were made by using Dunn’s method.

gene family homologues tested, the expression of *CpALS7* showed by far the largest increase in expression when grown in M199. When additional *C. parapsilosis* isolates (Table 1) were tested under the same growth conditions, considerable variability in *CpALS7* mRNA expression levels was observed. Because genomic heterogeneity among *C. parapsilosis* isolates has been noted (25), standard PCR was used with genomic DNA as the template to test for the presence of genomic sequences for each gene in each isolate. All of the wild-type isolates listed in Table 1 generated a PCR product for each gene, with the exception of strains JMB77 and Ro75. These two strains had a PCR product for all genes except *CPAR2\_404790*. To test the hypothesis that the level of expression of *CpALS7* is directly associated with adhesion, the *C. parapsilosis* isolates that showed variability in *CpALS7* expression levels were tested in adhesion assays under shear at 5 dynes/cm<sup>2</sup> (Fig. 6). Higher expression levels of *CpALS7* showed a strong



**FIG 4** Effect of serum on adhesion. *C. parapsilosis* strain JMB81 was induced by growth for 3 h at 37°C in either M199 or 100% fetal calf serum (FCS) and tested in adhesion assays in the presence or absence of fetal calf serum, as indicated. The adhesion index was calculated from duplicate channels with a minimum of 3 biological replicates. Statistical analysis was performed by using Kruskal-Wallis analysis of variance on ranks, and between-group comparisons were made by using Dunn’s method. \*,  $P < 0.001$ .



**FIG 5** Expression of *CpALS* genes during growth in M199. *C. parapsilosis* strain JMB81 was induced by growth in M199 for 3 h at 37°C, and qPCR was performed to evaluate the transcription of *ALS* gene homologues. Gene names are abbreviated as listed in Table 2. The relative expression level is shown for each gene from control cells (black bars) and induced cells (white bars). Error bars represent standard deviations from duplicate samples with 3 biological replicates. Comparisons of expression levels for each gene under control and induced conditions were made by a 2-tailed *t* test. \*,  $P < 0.001$  for induced versus control cells.

positive correlation with adhesion ( $R^2 = 0.71$ ;  $P < 0.001$ ), suggesting that *CpALS7* may have a direct role in the adhesion phenotype. None of the other *ALS* genes surveyed showed a positive or negative correlation with the adhesion index (see Fig. S1 in the supplemental material).

To test the relationship between *CpALS7* expression and adhesion in a more direct manner, the gene was cloned and expressed in *Saccharomyces cerevisiae* under the control of a galactose-inducible promoter (Fig. 7A). The induction of *CpALS7* expression in *S. cerevisiae* yeast cells led to significantly increased adhesion at 1 and 5 dynes/cm<sup>2</sup> relative to that of yeast cells that were grown with glucose as a carbon source ( $P < 0.001$ ). Yeast cells that contained the vector without *CpALS7* showed minimal adhesion regardless of the carbon source. To further investigate the effect of *CpALS7* on adhesion, a *CpALS7* deletion strain (KO) (19) and its wild-type parent (ATCC 22019) were

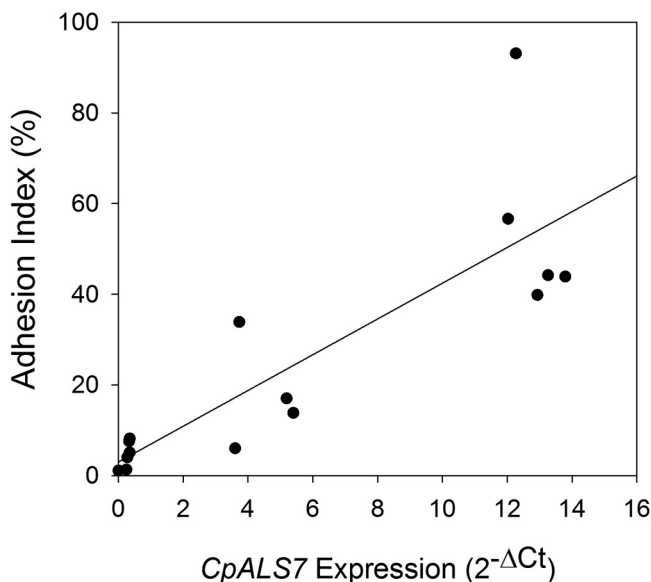
**TABLE 1** Strains and plasmids used in this study

Strain or plasmid	Description and/or genotype
<b>Strains</b>	
JMB72	Invasive clinical isolate from infant blood culture <sup>a</sup>
JMB77	Invasive clinical isolate from infant blood culture <sup>a</sup>
JMB81	Invasive clinical isolate from infant blood culture <sup>a</sup>
WIH04	Invasive clinical isolate from infant blood culture <sup>a</sup>
JMB317	Commensal clinical isolate from infant stool <sup>b</sup>
JMB318	Commensal clinical isolate from infant stool <sup>b</sup>
JMB319	Commensal clinical isolate from infant stool <sup>b</sup>
Ro75	Commensal clinical isolate from infant stool <sup>b</sup>
ATCC 22019	Wild type (parent of KO)
KO	<i>als7Δ::FRT/als7Δ::FRT</i> <sup>c</sup>
INVSc1	<i>S. cerevisiae</i> host strain for expression; <i>MATa his3D1 leu2 trp1-289 ura3-52 MAT his3D1 leu2 trp1-289 ura3-52</i>
JMB278	INVSc1 containing pYES2
JMB279	INVSc1 containing pBMJ44
<b>Plasmids</b>	
pYES2	Yeast expression vector (ThermoFisher Scientific)
pBMJ44	<i>CpALS7</i> under the control of a galactose-inducible promoter

<sup>a</sup>See reference 22.

<sup>b</sup>See reference 23.

<sup>c</sup>See reference 19.



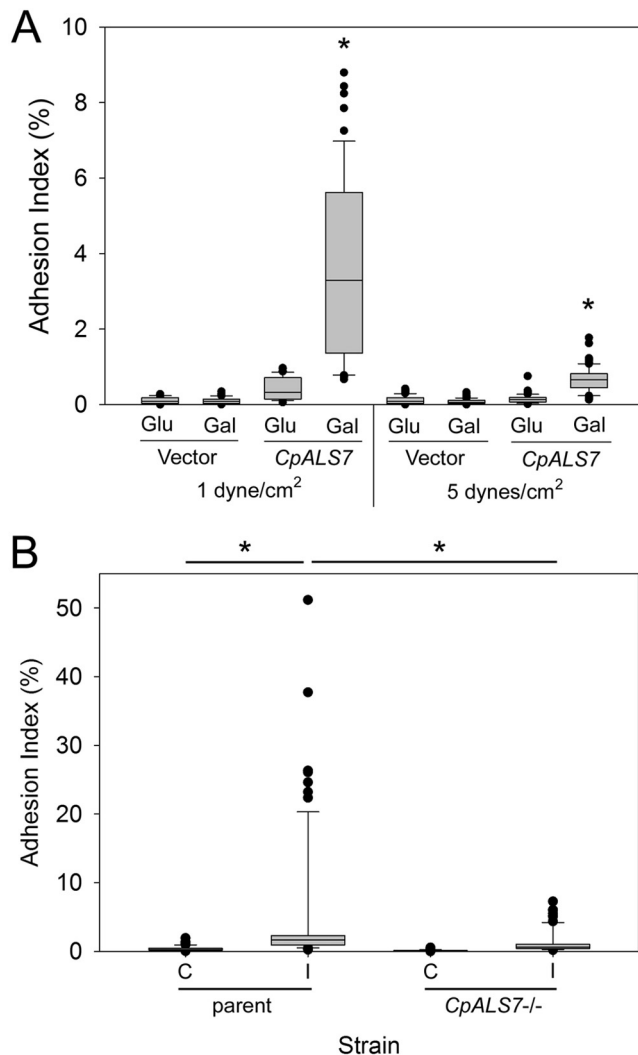
**FIG 6** Association of *CpALS7* expression with adhesion. *C. parapsilosis* isolates that showed various expression levels of *CpALS7* when induced by growth in M199 for 3 h at 37°C were tested in adhesion assays as described in the text. The adhesion index was plotted against the expression level of *CpALS7* measured by qPCR as described in the text. Linear regression analysis showed a strong positive correlation between *CpALS7* expression and adhesion ( $R^2 = 0.71$ ;  $P < 0.001$ ).

tested in the adhesion assay (Fig. 7B). Although the parent strain had low baseline levels of adhesion, induction in M199 medium resulted in a small increase. The induction of this strain also demonstrated a modest increase in the *CpALS7* RNA expression level relative to those of the other strains tested (not shown). Nevertheless, the *CpALS7* deletion strain had significantly reduced adherence in these assays. Taken together, these data suggest that *CpALS7* expression is an important component of the adhesion of *C. parapsilosis* to immobilized albumin under shear.

## DISCUSSION

Adhesion of *C. parapsilosis* to host surfaces remains poorly characterized relative to that of *C. albicans*. Investigation into the mechanisms by which *C. parapsilosis* adheres to the extracellular matrix (ECM) offers insight into the organism's underlying pathobiology and may provide a route to disrupt adhesion and prevent subsequent invasive infection. The objective of this study was to identify adhesins in *C. parapsilosis* that mediate binding to the extracellular matrix under physiologically relevant shear flow.

Because members of the *ALS* gene family in *C. albicans* have a role in adhesion, we screened for differential gene expression of *ALS* homologues under adhesive and nonadhesive conditions to identify putative adhesins in *C. parapsilosis*. The expression of *CpALS7* was markedly upregulated relative to other *ALS* gene homologues in strains that demonstrated inducible adhesion. A role for *CpAls7* in adhesion was first suggested by a study showing that the protein was isolated from a fibronectin affinity column (17). Another study showed that a strain deficient in *CpALS7* had reduced adhesion to buccal epithelial cells *in vitro* and a reduction in the fungal burden in a mouse model of urinary tract infection (19). Our study provides additional evidence to support a role for *CpAls7* in adhesion. First, we utilized an inducible expression system in *S. cerevisiae*, a well-established strategy to identify putative adhesins in *Candida* spp. (26). The induction of *CpALS7* expression in *S. cerevisiae* yielded a significant increase in adhesion in our assay system. However, the level of adhesion of the *S. cerevisiae* transformant remained relatively low compared to those of adhesive *C. parapsilosis* strains. It is possible that differences in codon usage contributed to the decreased functionality, as *C. parapsilosis* belongs to the CTG clade (where CTG is translated as serine rather than leucine as it is in *S. cerevisiae*). However, such discrepancies in



**FIG 7** Expression of *CpALS7* leads to increased adhesion. (A) *CpALS7* was cloned into the *S. cerevisiae* expression vector pYES2 under the control of the galactose-inducible promoter. Yeast cells containing this plasmid or the vector only were grown with either glucose (Glu) (repressive) or galactose (Gal) (inductive) as the sole carbon source, and adhesion was measured in the microfluidics device with a shear force of 1 or 5 dynes/cm<sup>2</sup>. Data are from duplicate samples at each shear force with 5 biological replicates. Comparisons between cells grown in Glu and those grown in Gal were made by using a Mann-Whitney rank sum test. \*,  $P < 0.001$  for Gal versus Glu at each shear force. (B) A *C. parapsilosis* strain harboring a homozygous deletion of *CpALS7* (*CpALS7*<sup>-/-</sup>) and the wild-type parent strain from which it was derived (19) were induced by growth in M199 for 3 h at 37°C and tested in adhesion assays as described above. The adhesion indices of induced (I) and control (C) yeast cells were calculated from duplicate channels with a minimum of 3 biological replicates. Statistical analysis was performed by using Kruskal-Wallis analysis of variance on ranks, and between-group comparisons were made by using Dunn's method. \*,  $P < 0.001$ .

adhesion are also likely to reflect inherent differences in the overall surface structures of *C. parapsilosis* and the generally nonadherent yeast *S. cerevisiae*.

Individual *C. parapsilosis* isolates displayed wide variability in the expression of *CpALS7* mRNA under inducing conditions. The variability in *CpALS7* expression and associated adhesion may reflect underlying genomic heterogeneity among strains of *C. parapsilosis*. Available genomic data collected from *C. parapsilosis* isolates have uncovered marked heterogeneity in general and variability in the presence and copy number of *ALS* genes in particular, reinforcing the understanding that *C. parapsilosis* is not clonal in its global distribution (25). Although we detected a PCR product for each *ALS* gene tested in each strain, except for *CPAR2\_404790* in 2 isolates, PCR analysis provides



only a rough assessment of the genomic heterogeneity that could affect gene expression. The possible underlying genomic variability among the isolates in our study is the subject of ongoing investigation. We used this variability to probe whether variation in *CpALS7* expression among different isolates could explain differences in the efficiency of adhesion to immobilized BSA in our flow adhesion assays. The strong positive correlation between the *CpALS7* mRNA level and the adhesion index provides further evidence of its role in adhesion.

The ability of induced yeast cells to adhere to fibronectin, collagen, and BSA under shear suggests that CpAls7 binds a wide spectrum of substrates. Binding to various substrates has also been reported for several *ALS* genes in *C. albicans* (6, 12), and recent efforts have provided insight into the molecular mechanisms involved (27, 28). Als proteins in *C. albicans* consist of four domains: an N-terminal (NT) domain that is the most conserved among Als proteins, a threonine-rich (T) region, a central region with a variable number of 36-amino-acid repeats, and a C-terminal domain that varies in size and contains a GPI anchor attaching the protein to the cell wall (6). The NT domain confers the adhesive function and contains a peptide-binding cavity (PBC) that has been resolved by X-ray crystallography (27). The PBC contains an invariant lysine residue that is required to interact with the C-terminal carboxyl group of a peptide ligand, and the structure and molecular interactions within the PBC account for the broad substrate specificity of these proteins. Examination of the deduced primary sequence of CpAls7 confirms that this protein has the same domain structure as that of the *C. albicans* Als family and bears the conserved lysine residue as well as the 8 cysteine residues that are responsible for the conserved disulfide bonds. As such, the mechanism of ligand binding is likely similar to that of *C. albicans*.

The preferential binding of *C. parapsilosis* to the ECM under fluid shear is reminiscent of microbial "catch bonding," a phenomenon in which tensile force applied to an environment increases the strength of bonds formed between microbes and/or between microbes and surfaces (29, 30). Recent work has established the involvement of *C. albicans* Als5 in catch bonding (31). Both wild-type *C. albicans* and *S. cerevisiae* expressing Als5 showed increased attachment as the shear force increased from 0.2 to 1.6 dynes/cm<sup>2</sup>. Higher shear force also led to more robust biofilm growth. The importance of the amyloid-forming region (AFR) of Als5 was demonstrated in this study. Additional work with Als3 has provided evidence that allows the separation of adhesion functions of Als3 (to protein ligands) from its role in the aggregation of fungal cells to each other (28). These data suggest that the AFR is more relevant for the latter. In our system, both adhesion to the ECM substrate and the aggregation of yeast cells to each other likely have a role in increasing the "adhesion index." Structural similarities predicted by the similar domain structure of CpAls7 suggest that similar mechanisms are likely to be at play, but confirmation will require experimental validation. Regardless, the preferential binding of *C. parapsilosis* to the ECM at 5 dynes/cm<sup>2</sup> may demonstrate the significance of this particular shear stress during adhesion to the ECM in the bloodstream or urinary tract. Given that physiological shear stress levels in capillaries and postcapillary venules are generally  $\leq 5$  dynes/cm<sup>2</sup>, these results suggest that initial adhesion is more likely to take place at these locales, as opposed to arterial fluid shear, which typically ranges from 10 to 30 dynes/cm<sup>2</sup> (32, 33).

We hypothesize that M199, as a mammalian cell culture medium, may partially mimic the host environment and induce adaptive changes in *C. parapsilosis*, analogous to the yeast-to-hypha switch of *C. albicans*. It should be noted, however, that the expression levels of *ALS* gene products in *C. albicans* can vary considerably under *in vitro* and *in vivo* conditions, which may limit the applicability of these findings (34). Adhesion also required the presence of Ca<sup>2+</sup> ions. Ca<sup>2+</sup> is present in plasma and tissue extracellular fluid, and the mammalian integrin, cadherin, and selectin families of adhesion molecules are dependent on the presence of millimolar levels of divalent cations. The requirement for Ca<sup>2+</sup> further suggests that CpAls7 may have evolved to provide adhesive functions in blood and tissues; however, it is not clear at present if divalent cations are required by the adhesin, the substrate, or both.

This study provides new and compelling evidence that CpAls7 aids in the adherence of *C. parapsilosis* to the ECM. CpAls7 may therefore represent an important inducible virulence factor for *C. parapsilosis* to adhere to blood vessel walls while resisting hemodynamic fluid shear and may play a role in the early stages of biofilm formation. Lessons learned not only are likely to be applicable to this specific host-pathogen interaction but also will better inform the pathogenic lifestyles of organisms that require a bloodstream phase.

## MATERIALS AND METHODS

**Strains and growth conditions.** The strains used in this study are listed in Table 1. *C. parapsilosis* strains were maintained in frozen stocks in 25% glycerol and recovered on YPD agar (1% yeast extract, 2% peptone, 2% dextrose). Parent strain ATCC 22019 and the homozygous *als7Δ/als7Δ* mutant (*CpALS7* KO) were graciously provided by Arianna Tavanti (19). Cultures for adhesion assays and qPCR were grown overnight with vigorous agitation in YPD broth at 37°C. *S. cerevisiae* strain INVSc1 was maintained as described above, with incubations at 30°C.

The *S. cerevisiae* transformants JMB278 and JMB279 (see below) were grown overnight in SC-URA (synthetic complete yeast agar without uracil) at 30°C with vigorous agitation. Following culture overnight, cells were washed with sterile water and adjusted to an optical density ( $A_{600}$ ) of 0.4 in fresh SC-URA supplemented with either 2% galactose or 2% glucose (to induce or repress expression under the control of the *GAL1* promoter, respectively). The cultures were incubated at 30°C for 24 h with vigorous agitation. Cultures were washed with HBSS<sup>+</sup> and adjusted to a concentration of  $3 \times 10^6$  yeast cells ml<sup>-1</sup> before introduction into the microfluidics adhesion assay mixture.

*C. parapsilosis* strains were grown overnight in YPD broth at 37°C with vigorous agitation. To achieve the adhesive phenotype, cultures grown overnight were washed with sterile water, resuspended in medium 199 (catalogue number BW12-117Q; BioWhittaker/Lonza) at a concentration of  $3 \times 10^6$  yeast cells ml<sup>-1</sup>, and incubated at 37°C for 3 to 6 h. This process is referred to as “induction.” Medium 199 was selected for induction because it efficiently induces hyphal growth and the expression of *ALS* genes important for adhesion in *C. albicans*. Medium 199 did not induce pseudohyphal growth of any *C. parapsilosis* strain studied under the conditions described here. Experiments involving amino acid supplements in HBSS<sup>+</sup> were conducted with the following concentrations: 225 μM L-aspartic acid, 455 μM L-glutamic acid, and 347 μM L-proline.

**Microfluidic adhesion assay.** Shear flow adhesion assays were conducted by using the Bioflux 200 system (Fluxion Biosciences) and Bioflux microchannel glass coverslip bottom plates at 37°C. This approach permitted running 24 samples in parallel under defined fluid shear and provided a medium-throughput assay for the simultaneous analysis of multiple conditions. Bioflux microchannels were coated with 2% BSA, 100 μg/ml fibronectin (R&D Systems), 800 μg/ml collagen type I (Sigma), or 1 mg/ml gelatin (Sigma) in HBSS<sup>+</sup> for 48 h. Microchannels were then washed with HBSS<sup>+</sup> at 2 dynes/cm<sup>2</sup> for 1 to 2 h to remove the unbound substrate. Yeast suspensions were introduced into each microchannel, and fluid flow was initiated for the entire plate. After 30 min of adhesion under fluid flow, yeast cells were aspirated from inlet and outlet wells without disturbing the channel area, DPBS<sup>+</sup> containing 5 μM calcofluor white (catalogue number F3543; Sigma) was added to microchannels, and fluid flow was resumed for another 10 min to wash away unbound yeast. Calcofluor is a fluorescent dye that binds to and stains yeast cell walls (Fig. 1A). The addition of calcofluor during washing (rather than during the adhesion step) reduced potential effects that the fluorescent dye binding may have on yeast adhesion. After washing, channels were imaged on a Nikon TE2000 inverted microscope using a Roper CoolSNAP HQ camera and Metavue 6.42r6 software (Molecular Devices). For some experiments, a Nikon Ti-E microscope with an Andor Zyla sCMOS camera and NIS-Elements software (Nikon) were used. On each microscope, a motorized stage was used to expedite the imaging of adjacent fields of view, permitting the programmed sequential capture of >80% of the channel length (Fig. 1B). Yeast adhesion was then calculated by using quantitative image analysis. Images were thresholded to differentiate brightly fluorescent yeast cells from dim background. A single threshold value was used per experiment so that all microchannels were analyzed with the same cutoff value. The thresholded area was measured by software and divided by the total imaged channel area to determine the percentage of the channel surface area covered by adherent yeast cells, defined as the adhesion index. This approach was developed because calcofluor fluorescence is dependent on pH and yeast strains. Measurement of the thresholded area minimized minor variations of the fluorescence intensity upon the calculation of adhesion efficiency. Microchannels were run in duplicate. The use of automated image capture and quantitative analysis reduced variability and contributed to the reproducible measurement of yeast adhesion. Data from a minimum of 3 independent experiments were pooled and were not normally distributed. Medians and percentile ranges were calculated, and comparisons between groups were made by a Mann-Whitney rank sum test or Kruskal-Wallis analysis of variance (ANOVA) on ranks, as indicated, with *P* values of <0.05 being considered significant. Statistical analysis was performed by using SigmaPlot version 13.0.

**RNA extraction, reverse transcription, and quantitative PCR.** RNA was isolated from cell pellets grown under the specified conditions, flash frozen in liquid nitrogen, and promptly stored at -80°C. Total RNA was extracted and purified by using the RiboPure Yeast extraction kit (Ambion/Life Technologies) according to the manufacturer's instructions. Prior to reverse transcription (RT), RNA samples were treated with DNase (Ambion/Life Technologies) to digest genomic DNA. RNA integrity was routinely

**TABLE 2** *C. parapsilosis* ALS and housekeeping gene homologues and primers

Gene	Abbreviation or alternate gene name	qPCR primer	Primer sequence
CPAR2_500660	500660	500660_F 500660_R	ATGGCTTGGATCAACCACTT CAGAACTTACGGCAGGGGTA
CPAR2_404770	404770	404770_F 404770_R	AACTGGCCATGAAAACAAGG CCATAGCAGGTTTGCACAGA
CPAR2_404790	404790	404790_F 404790_R	GCGTCAAACACAGAATCAA GCTGCTGTTGCTGTGACATT
CPAR2_404800	CpALS7	404800_F 404800_R	ACCACCACCGAGGTTACAAA TGATCTCCGGTTTTGAGTC
CPAR2_404780 <sup>a</sup>	404780	404780_F 404780_R	CGCAAGTACTCCTCCAACC CCATTACCTTCGTTCCATTG
CPAR2_201570	CpACT1	CpACT1_F CpACT1_R	CGAACGTGGTTACGGTTTCT TGACCATCTGGCAATTCGTA
CPAR2_502950	CpPMA1	CpPMA1_F CpPMA1_R	CGCCAACGAAGTTGTTCTG AGATTCACCAGTGATGGCGG
CPAR2_806050	CpRIP1	CpRIP1_F CpRIP1_R	CCCAATTGGTGAAGCAGGTG CCAAGTTCAAAGGGGCTGGA
CPAR2_807570	CpRPP2B	CpRPP2B_F CpRPP2B_R	CTCCCCATCTGCTTCAGACA TGAGGCCAATTTGGTGTACCT
CPAR2_204740	CpLSC2	CpLSC2_F CpLSC2_R	TTGGAAGTGGTGGTACTGC TTGCGGCAATCAAACCTTG
CPAR2_104580	CpIMH3	CpIMH3_F CpIMH3_R	TCACTGTTGGGGACGTCAAG GGATCGTCGTTGTTTCGTGA
CPAR2_401060	CpCPA1	CpCPA1_F CpCPA1_R	CCCGAGAGCATGACTGATCC AGCAACATCACCAACGACGA
CPAR2_808670	CpTDH3	CpTDH3_F CpTDH3_R	ACCACCAACTGTTGGCTCC ACCACCTCTCCAGTCCTTGT

<sup>a</sup>The primer set for CPAR2\_404780 was noted to also recognize CPAR2\_500660 during the review process. Analysis of qPCR data from samples containing these primer sets indicated that the expression level of CPAR2\_500660 was extremely low and contributed ~1% to the total mRNA levels at most. This observation is very consistent with previously reported data (19). Since the contribution of CPAR2\_500660 to the calculations of CPAR2\_404780 levels was negligible, the cross-hybridization of this primer set does not affect the data presented in a meaningful way.

monitored by agarose gel electrophoresis. cDNA was synthesized from 1  $\mu$ g total RNA with a blend of oligo(dT) and random hexamer primers using iScript reverse transcriptase (Bio-Rad). To verify the absence of genomic DNA, no-reverse-transcriptase controls were run in parallel with each sample.

Quantitative PCR was performed by using iTaq Universal SYBR green (Bio-Rad) and the ABI 7500 Fast system (Applied Biosystems). Primer sequences are listed in Table 2. All primers were used at a concentration of 500 nM. Primer specificity was determined via melting curve analysis and agarose gel electrophoresis. Controls lacking a template were routinely run to check for contamination or primer-dimer formation. Amplification efficiencies were assessed by using relative standard curves constructed from a cDNA dilution series. All efficiencies were estimated to be 92 to 100%. Given the sensitivity required for our experiments, we determined that the relative quantitation ( $2^{-\Delta\Delta C_T}$ ) method was suitable to calculate expression levels. The relative RNA expression level of each candidate gene was quantified by comparing cells grown in M199 (induced) to cells grown in YPD medium (control).

To identify a suitable reference gene for RT-qPCR analyses, a series of housekeeping gene homologues from *C. albicans* (*ACT1*, *PMA1*, *RIP1*, *RPP2B*, *LSC2*, *IMH3*, *TDH3*, and *CPA1*) was evaluated for expression stability (35). Relative transcript levels were examined under different growth conditions, as well as across different strains, by using RT-qPCR. According to the Normfinder algorithm (36), *ACT1* displayed the most stable expression profile and was therefore selected to be the reference gene for our studies.

**PCR amplification of CpALS genes.** To test for the presence of each ALS gene at the genomic level in the *C. parapsilosis* strains used in this study (Table 1), standard PCR was conducted by using genomic DNA from each strain as the template and the primers for each gene listed in Table 2. Reactions were

carried out by using Illustra Ready-To-Go PCR beads (GE Healthcare) under conditions recommended by the manufacturer. PCR products were analyzed by agarose gel electrophoresis.

**Cloning and expression of CpALS7 in *S. cerevisiae*.** A circular polymerase extension cloning (CPEC) strategy (37) was used to clone the *C. parapsilosis* CpALS7 gene under the control of the *GAL1* promoter in the yeast expression vector pYES2 (Invitrogen). The gene was PCR amplified from genomic DNA of JMB81 by using Phusion high-fidelity polymerase (Thermo Scientific) according to the manufacturer's instructions, with the following CPEC forward and reverse primers, respectively: 5'-GGCCGCCAGTGTGC GCACGAATCCAATGGTCAAAC-3' and 5'-GCCAGTGTGATGGATATCTGCAGTTAGATAACAAGAAAAGACTC AAGAC-3'. The product was purified through a spin column, and 100 ng was added to a CPEC reaction mixture with 100 ng pYES2 plasmid DNA linearized with EcoRI, 400  $\mu$ M deoxynucleoside triphosphates (dNTPs), and 1 U Phusion polymerase. The reaction mixture was heated in a thermocycler to 98°C for 3 min, followed by 10 cycles of 98°C for 30 s, 55°C for 30 s, and 72°C for 2.5 min, and the reaction was completed with a 10-min incubation at 72°C. The entire reaction mixture was transformed into competent *Escherichia coli* Top10 cells, and ampicillin-resistant transformants were screened with appropriate restriction digests. The expected sequence of the resulting plasmid (pBMJ44) was confirmed by DNA sequencing. pBMJ44 was transformed into *S. cerevisiae* INVSc1 by electroporation. Transformants containing an empty vector (JMB278) or CpALS7 (JMB279) were selected on SC-URA (Sigma) and subsequently cultured in SC-URA at 30°C.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00892-17>.

**SUPPLEMENTAL FILE 1**, PDF file, 0.2 MB.

## ACKNOWLEDGMENTS

We are grateful to Arianna Tavanti for providing the CpALS7 deletion strain.

Research reported in this publication was supported by an institutional development award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health (P30GM114750) and the Kilguss Research Core at Women & Infants Hospital of Rhode Island. It was also supported by the William and Mary Oh-William and Elsa Zopf Professorship in Pediatrics for Perinatal Research (S.K.S. and J.M.B.).

## REFERENCES

- Kullberg BJ, Arendrup MC. 2015. Invasive candidiasis. *N Engl J Med* 373:1445–1456. <https://doi.org/10.1056/NEJMra1315399>.
- Noyola DE, Fernandez M, Moylett EH, Baker CJ. 2001. Ophthalmologic, visceral, and cardiac involvement in neonates with candidemia. *Clin Infect Dis* 32:1018–1023. <https://doi.org/10.1086/319601>.
- Horn DL, Neofytos D, Anaissie EJ, Fishman JA, Steinbach WJ, Olyaei AJ, Marr KA, Pfaller MA, Chang CH, Webster KM. 2009. Epidemiology and outcomes of candidemia in 2019 patients: data from the prospective antifungal therapy alliance registry. *Clin Infect Dis* 48:1695–1703. <https://doi.org/10.1086/599039>.
- Thompson DS, Carlisle PL, Kadosh D. 2011. Coevolution of morphology and virulence in *Candida* species. *Eukaryot Cell* 10:1173–1182. <https://doi.org/10.1128/EC.05085-11>.
- Hofs S, Mogavero S, Hube B. 2016. Interaction of *Candida albicans* with host cells: virulence factors, host defense, escape strategies, and the microbiota. *J Microbiol* 54:149–169. <https://doi.org/10.1007/s12275-016-5514-0>.
- Hoyer LL, Cota E. 2016. *Candida albicans* agglutinin-like sequence (Als) family vignettes: a review of Als protein structure and function. *Front Microbiol* 7:280. <https://doi.org/10.3389/fmicb.2016.00280>.
- Hoyer LL. 2001. The ALS gene family of *Candida albicans*. *Trends Microbiol* 9:176–180. [https://doi.org/10.1016/S0966-842X\(01\)01984-9](https://doi.org/10.1016/S0966-842X(01)01984-9).
- Hoyer LL, Green CB, Oh SH, Zhao X. 2008. Discovering the secrets of the *Candida albicans* agglutinin-like sequence (ALS) gene family—a sticky pursuit. *Med Mycol* 46:1–15. <https://doi.org/10.1080/13693780701435317>.
- Phan QT, Myers CL, Fu Y, Sheppard DC, Yeaman MR, Welch WH, Ibrahim AS, Edwards JE, Filler SG. 2007. Als3 is a *Candida albicans* invasin that binds to cadherins and induces endocytosis by host cells. *PLoS Biol* 5:e64. <https://doi.org/10.1371/journal.pbio.0050064>.
- Fu Y, Phan QT, Luo G, Solis NV, Liu Y, Cormack BP, Edwards JE, Jr, Ibrahim AS, Filler SG. 2013. Investigation of the function of *Candida albicans* Als3 by heterologous expression in *Candida glabrata*. *Infect Immun* 81:2528–2535. <https://doi.org/10.1128/IAI.00013-13>.
- Almeida RS, Brunke S, Albrecht A, Thewes S, Laue M, Edwards JE, Filler SG, Hube B. 2008. The hyphal-associated adhesin and invasin Als3 of *Candida albicans* mediates iron acquisition from host ferritin. *PLoS Pathog* 4:e1000217. <https://doi.org/10.1371/journal.ppat.1000217>.
- Liu Y, Filler SG. 2011. *Candida albicans* Als3, a multifunctional adhesin and invasin. *Eukaryot Cell* 10:168–173. <https://doi.org/10.1128/EC.00279-10>.
- Pereira GH, Muller PR, Szesz MW, Levin AS, Melhem MS. 2010. Five-year evaluation of bloodstream yeast infections in a tertiary hospital: the predominance of non-*C. albicans* *Candida* species. *Med Mycol* 48:839–842. <https://doi.org/10.3109/13693780903580121>.
- Pfaller MA, Diekema DJ. 2004. Rare and emerging opportunistic fungal pathogens: concern for resistance beyond *Candida albicans* and *Aspergillus fumigatus*. *J Clin Microbiol* 42:4419–4431. <https://doi.org/10.1128/JCM.42.10.4419-4431.2004>.
- Trofa D, Gacser A, Nosanchuk JD. 2008. *Candida parapsilosis*, an emerging fungal pathogen. *Clin Microbiol Rev* 21:606–625. <https://doi.org/10.1128/CMR.00013-08>.
- Skrzypek MS, Binkley J, Binkley G, Miyasato SR, Simison M, Sherlock G. 2017. The *Candida* Genome Database (CGD): incorporation of assembly 22, systematic identifiers and visualization of high throughput sequencing data. *Nucleic Acids Res* 45:D592–D596. <https://doi.org/10.1093/nar/gkw924>.
- Kozik A, Karkowska-Kuleta J, Zajac D, Bochenska O, Kedracka-Krok S, Jankowska U, Rapala-Kozik M. 2015. Fibronectin-, vitronectin- and laminin-binding proteins at the cell walls of *Candida parapsilosis* and *Candida tropicalis* pathogenic yeasts. *BMC Microbiol* 15:197. <https://doi.org/10.1186/s12866-015-0531-4>.
- Karkowska-Kuleta J, Zajac D, Bras G, Bochenska O, Rapala-Kozik M, Kozik A. 2017. Binding of human plasminogen and high-molecular-mass kininogen by cell surface-exposed proteins of *Candida parapsilosis*. *Acta Biochim Pol* 64:391–400. [https://doi.org/10.18388/abp.2017\\_1609](https://doi.org/10.18388/abp.2017_1609).
- Bertini A, Zoppo M, Lombardi L, Rizzato C, De Carolis E, Vella A, Torelli R,

- Sanguinetti M, Tavanti A. 2016. Targeted gene disruption in *Candida parapsilosis* demonstrates a role for *CPAR2\_404800* in adhesion to a biotic surface and in a murine model of ascending urinary tract infection. Virulence 7:85–97. <https://doi.org/10.1080/21505594.2015.1112491>.
20. Maguire SL, OhEigeartaigh SS, Byrne KP, Schroder MS, O'Gaora P, Wolfe KH, Butler G. 2013. Comparative genome analysis and gene finding in *Candida* species using CGOB. Mol Biol Evol 30:1281–1291. <https://doi.org/10.1093/molbev/mst042>.
21. Fitzpatrick DA, O'Gaora P, Byrne KP, Butler G. 2010. Analysis of gene evolution and metabolic pathways using the *Candida* Gene Order Browser. BMC Genomics 11:290. <https://doi.org/10.1186/1471-2164-11-290>.
22. Bliss JM, Wong AY, Bhak G, Laforce-Nesbitt SS, Taylor S, Tan S, Stoll BJ, Higgins RD, Shankaran S, Benjamin DK, Jr. 2012. *Candida* virulence properties and adverse clinical outcomes in neonatal candidiasis. J Pediatr 161:441.e2–447.e2. <https://doi.org/10.1016/j.jpeds.2012.02.051>.
23. Bliss JM, Basavegowda KP, Watson WJ, Sheikh AU, Ryan RM. 2008. Vertical and horizontal transmission of *Candida albicans* in very low birth weight infants using DNA fingerprinting techniques. Pediatr Infect Dis J 27:231–235. <https://doi.org/10.1097/INF.0b013e31815bb69d>.
24. Butler G, Rasmussen MD, Lin MF, Santos MA, Sakthikumar S, Munro CA, Rheinbay E, Grabherr M, Forche A, Reedy JL, Agrafioti I, Arnaud MB, Bates S, Brown AJ, Brunke S, Costanzo MC, Fitzpatrick DA, de Groot PW, Harris D, Hoyer LL, Hube B, Klis FM, Kodira C, Lennard N, Logue ME, Martin R, Neiman AM, Nikolaou E, Quail MA, Quinn J, Santos MC, Schmitzberger FF, Sherlock G, Shah P, Silverstein KA, Skrzypek MS, Soll D, Staggs R, Stansfield I, Stumpf MP, Sudbery PE, Srikantha T, Zeng Q, Berman J, Berriman M, Heitman J, Gow NA, Lorenz MC, Birren BW, Kellis M, et al. 2009. Evolution of pathogenicity and sexual reproduction in eight *Candida* genomes. Nature 459:657–662. <https://doi.org/10.1038/nature08064>.
25. Prysacz LP, Nemeth T, Gacsér A, Gabaldón T. 2013. Unexpected genomic variability in clinical and environmental strains of the pathogenic yeast *Candida parapsilosis*. Genome Biol Evol 5:2382–2392. <https://doi.org/10.1093/gbe/evt185>.
26. Sheppard DC, Yeaman MR, Welch WH, Phan QT, Fu Y, Ibrahim AS, Filler SG, Zhang M, Waring AJ, Edwards JE, Jr. 2004. Functional and structural diversity in the Als protein family of *Candida albicans*. J Biol Chem 279:30480–30489. <https://doi.org/10.1074/jbc.M401929200>.
27. Salgado PS, Yan R, Taylor JD, Burchell L, Jones R, Hoyer LL, Matthews SJ, Simpson PJ, Cota E. 2011. Structural basis for the broad specificity to host-cell ligands by the pathogenic fungus *Candida albicans*. Proc Natl Acad Sci U S A 108:15775–15779. <https://doi.org/10.1073/pnas.1103496108>.
28. Lin J, Oh SH, Jones R, Garnett JA, Salgado PS, Rusnakova S, Matthews SJ, Hoyer LL, Cota E. 2014. The peptide-binding cavity is essential for Als3-mediated adhesion of *Candida albicans* to human cells. J Biol Chem 289:18401–18412. <https://doi.org/10.1074/jbc.M114.547877>.
29. Isberg RR, Barnes P. 2002. Dancing with the host; flow-dependent bacterial adhesion. Cell 110:1–4. [https://doi.org/10.1016/S0092-8674\(02\)00821-8](https://doi.org/10.1016/S0092-8674(02)00821-8).
30. Thomas W. 2008. Catch bonds in adhesion. Annu Rev Biomed Eng 10:39–57. <https://doi.org/10.1146/annurev.bioeng.10.061807.160427>.
31. Chan CX, Lipke PN. 2014. Role of force-sensitive amyloid-like interactions in fungal catch bonding and biofilms. Eukaryot Cell 13:1136–1142. <https://doi.org/10.1128/EC.00068-14>.
32. dela Paz NG, D'Amore PA. 2009. Arterial versus venous endothelial cells. Cell Tissue Res 335:5–16. <https://doi.org/10.1007/s00441-008-0706-5>.
33. Shaik SS, Soltan TD, Chaturvedi G, Totapally B, Hagood JS, Andrews WW, Athar M, Voitenok NN, Killingsworth CR, Patel RP, Fallon MB, Maheshwari A. 2009. Low intensity shear stress increases endothelial ELR<sup>+</sup> CXCL chemokine production via a focal adhesion kinase-p38 $\beta$  MAPK-NF- $\kappa$ B pathway. J Biol Chem 284:5945–5955. <https://doi.org/10.1074/jbc.M807205200>.
34. Coleman DA, Oh SH, Manfra-Maretta SL, Hoyer LL. 2012. A monoclonal antibody specific for *Candida albicans* Als4 demonstrates overlapping localization of Als family proteins on the fungal cell surface and highlights differences between Als localization in vitro and in vivo. FEMS Immunol Med Microbiol 64:321–333. <https://doi.org/10.1111/j.1574-695X.2011.00914.x>.
35. Nailis H, Coenye T, Van Nieuwerburgh F, Deforce D, Nelis HJ. 2006. Development and evaluation of different normalization strategies for gene expression studies in *Candida albicans* biofilms by real-time PCR. BMC Mol Biol 7:25. <https://doi.org/10.1186/1471-2199-7-25>.
36. Andersen CL, Jensen JL, Orntoft TF. 2004. Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets. Cancer Res 64:5245–5250. <https://doi.org/10.1158/0008-5472.CAN-04-0496>.
37. Quan J, Tian J. 2014. Circular polymerase extension cloning. Methods Mol Biol 1116:103–117. [https://doi.org/10.1007/978-1-62703-764-8\\_8](https://doi.org/10.1007/978-1-62703-764-8_8).