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<td>This is a pre-copyedited, author-produced PDF of an article accepted for publication in FEMS yeast research following peer review. The version of record FEMS Yeast Res. 2014 Jun;14(4):674-7. Okamoto-Shibayama K. et al is available online at: <a href="http://dx.doi.org/10.1111/1567-1364.12160">http://dx.doi.org/10.1111/1567-1364.12160</a>.</td>
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Csa2, a member of the Rbt5 protein family, is involved in the utilization of iron from human hemoglobin during Candida albicans hyphal growth

Running Head: Csa2 in iron utilization for C. albicans hyphal growth

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Csa2 is a member of both the *Candida albicans* Rbt5 protein family and the CFEM (Common in Fungal Extracellular Membranes) protein superfamily. CFEM proteins are characterized by an internal domain containing eight equally spaced cysteine residues. Csa2 is involved in iron uptake from hemoglobin and heme proteins; however, its precise role is unclear. Here, we provide quantitative evidence of the involvement of Csa2 in the utilization of iron from human hemoglobin during *C. albicans* hyphal growth. The ability of the hyphal form of the wild-type, a homozygote csa2Δ mutant, and a complemented strain of *C. albicans* to utilize hemoglobin as an iron source under iron-restricted conditions was examined through growth studies and a crystal violet-staining assay. Hemoglobin-binding activity was assessed indirectly by using a hemoglobin-sensitized tube method. Although hyphal growth of the wild-type and csa2Δ/Δ::CSA2 strains was completely recovered when a high concentration of human hemoglobin was added to the iron-restricted culture medium, the recovery of the csa2Δ/Δ mutant was significantly diminished. Furthermore, hemoglobin binding was impaired in the csa2Δ/Δ mutant compared with the wild-type and csa2Δ/Δ::CSA2 strains, revealing that Csa2 is involved in the utilization of hemoglobin as an iron source by the hyphal form of *C. albicans*.

**Keywords:** *Candida albicans*; hemoglobin; hyphal growth; iron utilization; Rbt5 protein family
*Candida albicans* is a major fungal pathogen that causes mucosal and systemic infections in immunocompromised hosts. A key factor in the virulence of *C. albicans* is its ability to switch from a yeast to a hyphal form while inside a host. This switch has been implicated in the pathogenesis of systemic *C. albicans* infections, because mutants with defective hyphal growth show reduced virulence (Lo *et al.*, 1997; Jacobsen *et al.*, 2012). The hyphal form of *C. albicans* is able to use hemoglobin as a source of iron, suggesting that this is an important factor in the switch to the hyphal form and in injury to the host. Because hemoglobin promotes true hyphal morphogenesis, hemoglobin utilization is considered a virulence factor (Tanaka *et al.*, 1997; Pendrak & Roberts, 2007).

*Candida albicans* has three main systems for acquiring iron: a reductive system, a siderophore uptake system, and a heme iron uptake system. The reductive pathway involves the release of iron from transferrin or ferritin or the exploitation of free iron in the environment. The glycosylphosphatidylinositol (GPI)-anchored cell-wall protein Als3 is a receptor for ferritin in the host environment (Almeida *et al.*, 2009). *Candida albicans* does not secrete siderophores, which are high-affinity iron chelators, to scavenge iron, but instead it takes up siderophores synthesized by other microorganisms via the Sit1 transporter (Heymann *et al.*, 2002). Independent of these two iron uptake systems, *C. albicans* is also capable of taking up iron from hemoglobin and heme proteins through a process of erythrocyte lysis, hemoglobin binding, heme extraction, and endocytosis (Manns *et al.*, 1994;
Weissman & Kornitzer, 2004). It has been suggested that the members of the *C. albicans* heme-receptor protein family that possess the CFEM (Common in Several Fungal Extracellular Membranes) domain (i.e., Csa1, Csa2 and Pga7 [Rbt6], Rbt5, and Rbt51 [Pga10]) are involved in this third system of iron uptake (Weissman & Kornitzer, 2004). Unlike the other CFEM-containing proteins, which are attached to both the cell wall and the plasma membrane, the small, secretory protein Csa2 does not possess a GPI anchor (Sosinska *et al.*, 2008; Weissman *et al.*, 2008; Sorgo *et al.*, 2010, 2011). Transcriptional studies have indicated that several cell wall proteins are regulated by iron availability, and that the increased expression of CFEM-containing proteins in conditions of iron starvation supports their involvement in iron acquisition (Lan *et al.*, 2004; Chen *et al.*, 2011). The precise roles of these heme-binding proteins in virulence have not yet been well described; in particular, little is known about the function of Csa2 in heme-iron uptake. Therefore, we aimed to clarify the involvement of Csa2 in iron acquisition during hyphal growth in *C. albicans*.

A *csa2Δ/Δ* mutant, previously described as *Δorf19.3117*, and a triple-auxotrophic strain, BWP17, complemented with plasmid CIp30, which was used as the wild-type control in all experiments, were kindly provided by Professor B. Hube of Hans Knoell Institute, Germany (Zakikhany *et al.*, 2007). A *csa2Δ/Δ::CSA2* complemented strain was constructed by introducing the wild-type allele of the *CSA2* gene into the RP10 locus of the *csa2Δ/Δ* mutant by using the CIp10 plasmid; the integration was verified by means of polymerase chain
reaction and DNA sequencing analyses.

Iron-restricted culture conditions were used as described (Weissman et al., 2008). Yeast cells were cultured at 30°C in YPD medium (1% yeast extract, 2% peptone, and 2% glucose per liter) supplemented with 1 mM ferrozine (an iron chelator) to elicit iron starvation. To induce switching to the hyphal form, yeast cells (1 × 10⁴ cells/mL) were transferred to RPMI 1640 medium in which free iron had been removed by the addition of 100 µg/mL of apotransferrin, and then cultured for 16 to 24 h under 5% CO₂ at 37°C.

Preliminary experiments showed that the growth of all three strains in RPMI 1640 medium was similar. All three strains also exhibited similar dose-dependent growth inhibition by chelating free iron, which is essential for growth, when incubated with a series of apotransferrin dilutions in RPMI 1640 medium. Growth was totally inhibited by the addition of 100 µg/mL apotransferrin; therefore, this concentration of apotransferrin was used to chelate the free iron in the culture medium. Hyphal growth morphology was confirmed microscopically and growth was assessed by means of a crystal violet (CV)-staining assay. Viable Candida hyphal cells were quantified by measuring the photometric absorbance of the Candida-bound CV extract at a wavelength of 590 nm (Abe et al., 1994).

Figure 1(a) shows the inhibitory effects of apotransferrin on hyphal growth and the rescue of hyphal growth by FeCl₃. All three strains exhibited a similar growth pattern when incubated in RPMI 1640 medium without 100 µg/mL apotransferrin. Hyphal growth in the wild-type or
csa2ΔΔ::CSA2 complemented strain was completely rescued by the addition of FeCl₃, but only partially so in the csa2ΔΔ mutant, indicating that Csa2 may play a role in non-hemoglobin iron utilization.

Next, we examined whether *C. albicans* was able to utilize hemoglobin as an iron source under iron-restricted conditions and whether the addition of human hemoglobin to the environment was able to rescue impaired hyphal growth (Fig. 1b). With the addition of human hemoglobin, hyphal growth was restored in the wild-type and csa2ΔΔ::CSA2 complemented strain but only partially so in the csa2ΔΔ mutant. The csa2ΔΔ mutant grew at approximately one-third the rate of the wild-type strain when a high concentration of human hemoglobin was the sole source of iron. These results indicate that Csa2 is involved in the utilization of human hemoglobin as a source of iron. Interestingly, deletion of CSA2 did not fully attenuate growth, implying that additional genes and mechanisms are involved in the exploitation of environmental iron.

In a hemoglobin binding assay, to quantify the adherent *C. albicans*, we used hemoglobin-sensitized tubes that were prepared by incubation overnight at 37°C with human hemoglobin in Ca²⁺- and Mg²⁺-free phosphate-buffered saline, as described previously (Tanaka *et al.*, 1997). We observed attenuation of the capacity of the csa2ΔΔ mutant to bind human hemoglobin compared with that of wild-type *C. albicans* (Fig. 1c).

We also examined whether Csa2 was involved in hemolysis by measuring the hemolytic
activity of the *C. albicans* culture supernatant. Culture supernatants from the three strains of *C. albicans* were mixed with human erythrocytes and incubated for 12 to 72 h at 37°C, and the hemolytic activity was measured by the absorption at 405 nm (Tanaka et al., 1997). No significant differences in hemolytic activity between the culture supernatant of wild-type *C. albicans* and that of the csa2Δ/Δ mutant were observed (data not shown), indicating that Csa2 was not involved in hemolysis.

Taken together, our results show that Csa2 is involved in iron utilization and hemoglobin binding, and that Csa2 binds to hemoglobin before presenting it to other heme-binding proteins.

Although the functions of the proteins in the Rbt5 family that possess the conserved CFEM are largely unknown, they are suspected to play important roles in fungal pathogenesis (Kulkarni et al., 2003). Sorgo et al. (2013) used mass spectrometry to characterize the dynamics of the response of the *C. albicans* cell-wall proteome to iron starvation, and in particular the remarkable changes in the levels of the GPI-modified members of the Rbt5 family; their results suggest that the CFEM domain is responsible for the heme-binding properties of these proteins. Furthermore, secretome analyses have shown that the level of Csa2 increases under conditions of iron starvation (Sorgo et al., 2013). Interestingly, in addition to using hemoglobin as an iron source, *C. albicans* also uses hemoglobin as a signaling molecule to alter gene expression and induce adhesion to several extracellular
matrix proteins (Pendrak & Roberts, 2007).

Further studies are required to delineate the molecular basis of iron uptake by *C. albicans*. Our results suggest that members of the Rbt5 protein family collaborate in iron acquisition and that Csa2 may bind to hemoglobin before presenting it to other heme-binding proteins. Our group is currently attempting to characterize the involvement of Csa2 in iron acquisition and virulence. In conclusion, our findings suggest that Csa2 is involved in the utilization of iron from human hemoglobin during hyphal growth in *C. albicans*.

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References


Figure legend

Fig. 1. Involvement of Csa2 in iron utilization during hyphal growth in *Candida albicans*.

(a) Inhibitory effects of transferrin on *C. albicans* hyphal growth and the restoration of hyphal growth by the addition of FeCl₃. *Candida albicans* (1 × 10⁴ cells/mL in RPMI 1640 medium containing 100 µg/mL apotransferrin) was cultured with various concentrations of FeCl₃ (0.01 to 1000 µg/mL). Hyphal growth was measured by means of a crystal violet (CV)-staining assay. Three independent experiments were performed in duplicate. Data are presented as mean ± S.D. wt, wild-type. (b) Rescue of *C. albicans* hyphal growth by the addition of human hemoglobin. *Candida albicans* (1 × 10⁴ cells/mL in RPMI 1640 medium containing 100 µg/mL apotransferrin) was cultured with various concentrations of human hemoglobin (0.1 to 1000 µg/mL). Hyphal growth was measured by means of a CV-staining assay. Three independent experiments were performed in duplicate. Data are presented as mean ± S.D. Student’s *t*-test, *P* < 0.001 vs. wild-type (wt). (c) Binding activity of *C. albicans* to human hemoglobin. *Candida albicans* (1 × 10⁴ cells/mL) was cultured in human hemoglobin–sensitized tubes containing RPMI 1640 medium for 24 h. Student’s *t*-test, *P* < 0.001 vs. adherent wild-type (wt) control cells.
FeCl$_3$ (µg/mL)
Hyphal growth (OD$_{590}$)

Fig. 1(a)

Human hemoglobin (µg/mL)
Hyphal growth (OD$_{590}$)

Fig. 1 (b)
Fig. 1 (c)