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FEBS J. Author manuscript; available in PMC 2017 February 01.

Published in final edited form as:

FEBS J. 2016 February ; 283(4): 619–633. doi:10.1111/febs.13389.

Histoplasma capsulatum surmounts obstacles to intracellular pathogenesis

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Abstract

The fungal pathogen *Histoplasma capsulatum* causes respiratory and disseminated disease, even in immunocompetent hosts. In contrast to opportunistic pathogens, which are readily controlled by phagocytic cells, *H. capsulatum* yeasts are able to infect macrophages, survive antimicrobial defenses, and proliferate as an intracellular pathogen. In this review, we discuss some of the molecular mechanisms that enable *H. capsulatum* yeasts to overcome obstacles to intracellular pathogenesis. *H. capsulatum* yeasts gain refuge from extracellular obstacles such as antimicrobial lung surfactant proteins by engaging the β-integrin family of phagocytic receptors to promote entry into macrophages. In addition, *H. capsulatum* yeasts conceal immunostimulatory β-glucans to avoid triggering signaling receptors such as the β-glucan receptor Dectin-1. *H. capsulatum* yeasts counteract phagocyte-produced reactive oxygen species by expression of oxidative stress defense enzymes including an extracellular superoxide dismutase and an extracellular catalase. Within the phagosome, *H. capsulatum* yeasts block phagosome acidification, acquire essential metals such as iron and zinc, and utilize *de novo* biosynthesis pathways to overcome nutritional limitations. These mechanisms explain how *H. capsulatum* yeasts avoid and negate macrophage defense strategies and establish a hospitable intracellular niche, making *H. capsulatum* a successful intracellular pathogen of macrophages.

Keywords

cell wall; CR3; Dectin-1; fungal pathogenesis; glucan; iron; phagosome; ROS; zinc

Introduction

Histoplasma capsulatum is a member of a group of fungal pathogens that are characterized by thermal dimorphism and cause respiratory and disseminated disease in mammals [1–3]. In the environment, *H. capsulatum* grows as a saprobic conidia-producing mycelium. When *H. capsulatum*-contaminated soil is disturbed, the aerosolized mycelium fragments and conidia may be inhaled, and the small size of the conidia enables them to reach the lower

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respiratory tract. *H. capsulatum* cells respond to the elevated temperature in the mammalian host by activation of a transcriptional program that induces a switch to the yeast-phase morphology and expression of factors required for pathogenesis [4–7]. *H. capsulatum* is classified as a primary pathogen [8] due to its ability to cause disease in normal (i.e. immunocompetent) hosts, in contrast to opportunistic fungal pathogens that require some deficiency of host immune function (e.g. neutropenia, AIDS, etc.).

Factors produced by *H. capsulatum* yeast cells enable them to parasitize phagocytic immune cells. Pathogenic-phase yeasts are capable of invading phagocytic cells, including alveolar macrophages, polymorphonuclear leukocytes and dendritic cells [9–11]. These phagocytes serve as both the host cell and the vector by which infection dissemination is mediated. The innate immune response alone is insufficient to control *H. capsulatum* infection, in contrast to the ability of innate cells to control and eliminate some opportunistic fungal pathogens (e.g. *Candida* and *Aspergillus* species). Un-impaired by the innate immune system, continued proliferation of *H. capsulatum* yeasts exacerbates disease symptoms and leads to infection of extra-pulmonary sites. Upon activation of cell-mediated immunity, pro-inflammatory cytokines produced primarily by $CD4^+$ T cells potentiate the antifungal properties of phagocytes and resolution of the *H. capsulatum* infection may occur. However, if an adequate T-cell response does not develop due to immuno-compromised conditions (e.g. HIV, anti-cytokine therapy, etc.), or as a result of larger inocula that establish high fungal burdens before activation of cell-mediated immunity, a life-threatening condition ensues [12–15]. Research over the past two decades has revealed a number of factors produced by *H. capsulatum* that subvert innate defenses and facilitate intracellular proliferation of the yeasts. In this review, we discuss findings that provide a molecular explanation of the mechanisms underlying *H. capsulatum*'s ability to surmount obstacles to infection and to survive within immune cells that are normally not conducive to fungal survival.

Extracellular obstacles to infection

Histoplasma capsulatum is almost exclusively found as an intracellular pathogen (Fig. 1). The species was so named by Samuel Darling because he observed it within phagocytic cells: 'histo-' because the microbe was found within histiocytes (a general term for tissue phagocytes), and '-plasma' because he believed the microbe was a parasite-like creature. To secure intra-cellular residence, *H. capsulatum* must first overcome extracellular obstacles to infection (Fig. 2).

Surfactant proteins

The paucity of extracellular organisms suggests that intracellular residence is not only characteristic of, but also beneficial to, *H. capsulatum* yeasts. Indeed, the phagocyte may provide a refuge for *H. capsulatum*, limiting exposure to soluble extracellular antimicrobial factors. Within the alveolar space, the initial site of *H. capsulatum* infection, the extracellular environment composition includes surfactant fluid. The surfactant proteins A and D (SP-A and SP-D) present in this lipid-rich fluid have been shown to have host defense functions [16]. SP-A and SP-D are pulmonary col-lectins that comprise a collagen-like region and a C-type lectin domain that permits binding to polysaccharides of microbes. *In vitro*, both SP-A

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and SP-D have direct antifungal effects on *H. capsulatum* yeasts at physiological concentrations, with SP-D showing slightly more fungicidal activity in short-term assays [17]. Both SP-A and SP-D cause permeabilization of *H. capsulatum* yeast cells, which accounts for their fungicidal activity. Internalization of *H. capsulatum* yeasts by alveolar macrophages completely negates the fungicidal activity of SP-A and SP-D, indicating that alveolar macrophages shelter *H. capsulatum* from the antimicrobial effects of surfactant proteins. Interestingly, SP-A and SP-D are not active against *Blastomyces dermatitidis*, yeasts that are more commonly found extracellularly and probably have mechanisms to resist SP-A and SP-D [17]. Despite the sensitivity of *H. capsulatum* yeasts to surfactant *in vitro*, the loss of SP-A in outbred mice only slightly enhanced *H. capsulatum* infection and dissemination *in vivo* during acute stages (day 7), suggesting that *H. capsulatum* yeasts may minimize exposure to SP-A *in vivo*, presumably through refuge in alveolar macrophages [17].

Internalization by host phagocytes

Histoplasma capsulatum yeasts are internalized by host phagocytes through induction of phagocytosis. Blocking phagocytosis by inhibition of the actin cytoskeleton of phagocytes prevents yeast internalization [18,19]. Complement receptors appear to be the major phagocytic receptors for binding and uptake of *H. capsulatum* yeasts (Fig. 2), and this internalization does not require opsonization by complement [18,20,21]. These receptors include LFA-1 (CD11a/ CD18), CR3 (CD11b/CD18; Mac-1) and CR4 (CD11c/CD18), which are expressed on alveolar and interstitial macrophages as well as polymorphonuclear leukocytes and dendritic cells. Antibody blocking of the common CD18 subunit prevents 50–90% of binding and uptake of *H. capsulatum* yeast by human macrophages [18,20], indicating that the majority of *H. capsulatum* uptake is dependent on the complement receptors. Yeast binding to macrophages requires Ca^{2+} and Mg^{2+} ions, and is temperature-sensitive [20], consistent with the divalent cation-dependent structural dynamics of integrin binding properties [22]. Treatment of macrophages with soluble mannose or β-glucans did not prevent or minimally reduced binding, suggesting that CR3 interaction is sufficient for yeast adhesion [19,20]. On the other hand, binding of yeasts to stimulated murine macrophages was only partially reduced by antibodies to complement receptors, suggesting the possible existence of additional phagocytic receptors on murine cells [19].

The *H. capsulatum* Hsp60 protein was identified as the ligand for CD11/CD18 integrin-type receptors by purification of *H. capsulatum* proteins binding to CR3 [21]. Although Hsp60 is a canonical intracellular chaperone protein, it has also been found localized to the surface of *H. capsulatum* yeast cells; antibodies to *H. capsulatum* Hsp60 detect Hsp60 protein in cell-wall extracts and label non-permeabilized yeast cells [23], and vaccination with Hsp60 provides the host with a protective immune response [24]. In addition, recombinant Hsp60 may competitively block adhesion of yeasts to macrophages [21,25]. Thus, the Hsp60–complement receptor interaction appears to be a major ligand–receptor pairing mediating adhesion to and internalization of *H. capsulatum* yeasts by macrophages. How normally intracellular Hsp60 becomes localized to the cell surface and whether it serves additional non-adhesion functions is not understood at this time. Similar to *H. capsulatum*, the related dimorphic fungal pathogen *Blastomyces dermatitidis* expresses a CR3-interacting protein,

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Bad1. *B. dermatitidis* Bad1 may mediate adherence to macrophages, suggesting that CR3 may be a shared entry mechanism [26]. However, *B. dermatitidis* yeasts expressing Bad1, but lacking it on the yeast surface, retain virulence, indicating that Bad1-mediated adhesion of yeast to macrophages is not required for *B. dermatitidis* pathogenesis [27].

Host pattern recognition receptors

The close interaction of *H. capsulatum* yeasts with the macrophage surface to promote internalization risks engagement of pattern recognition receptors that may stimulate immune responses detrimental to *H. capsulatum* yeasts. Phagocytosis mediated by CR3 in the absence of other activating receptor interactions is generally non-inflammatory as there is a lack of additional danger signals. *H. capsulatum*'s success in becoming intracellular thus hinges on minimization of activation of macrophage signaling receptors while simultaneously triggering phagocytic receptors. Signaling receptors that recognize fungal pathogen-associated molecular patterns (PAMPs) include the Toll-like receptors (TLRs), the macrophage mannose receptor and C-type lectin receptors [28]. Most of these receptors contain a carbohydrate recognition domain that is capable of binding to molecules of the glycan-and glycoprotein-rich cell walls that comprise the surface of fungal cells.

Dectin-1 is the primary receptor for detection of fungal β-glucans [29]. β-glucans serve as a central component of the cell wall for many fungi [30], and, when bound to Dectin-1, induce an inflammatory response that may include increased production of reactive oxygen species and release of pro-inflammatory cytokines [31–34]. The *H. capsulatum* cell wall contains abundant β -glucans that have the potential to activate Dectin-1 [35,36]. However, mice lacking Dectin-1 do not have increased susceptibility to *H. capsulatum* infection [37]. Dectin-1-deficient mice have slightly higher, but not statistically increased, pulmonary fungal burdens after *H. capsulatum* infection, although this was measured after 13 days, corresponding to the adaptive immunity phase [38]. Together, these studies indicate little, if any, role for Dectin-1 in control of *H. capsulatum* infection, suggesting that *H. capsulatum* yeast avoids interaction with Dectin-1. Molecularly, most strains of *H. capsulatum* accomplish this by surrounding the yeast cell with non-immunostimulatory α-linked glucans that effectively conceal the β-glucans and drastically reduce yeast recognition by Dectin-1 [39]. Cell wall α-glucan is synthesized by an α- (1,3)-glucan synthase (Ags1) that is thought to extend β-glucan assembly from short α- (1,4)-linked glucan oligomers formed by the Amy1 α-amylase-like protein [40]. Ags1 and Amy1 are both required for *H. capsulatum* α-glucan synthesis [41,42]. In contrast to the avirulent mycelia, only *H. capsulatum* yeast cells produce α-glucan [36], supporting a pathogenesis-specific function. Consistent with this, loss of α-glucan by depletion of Ags1 or Amy1 functions markedly attenuates *H. capsulatum* virulence *in vivo* [41,42]. Curiously, *H. capsulatum* isolates belonging to the North American type 2 phylogenetic group naturally lack α-glucan (chemotype II strains) [43], and show variable recognition by Dectin-1 *in vitro*, depending on yeast growth conditions [35,38]. Depletion of Ags1 function has no effect on the virulence of these strains [35]. The molecular mechanism by which North American type 2 strains have circumvented the need for α-glucan remains unknown. Regardless of the mechanism, one strategy used by *H. capsulatum* yeasts is minimization of β-glucan exposure, which enables stealthy interaction of yeasts with macrophages without triggering Dectin-1 signaling (Fig. 2). *B.*

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dermatitidis and *Paracoccidioides* species also contain α-glucan in the yeast phase [44,45], presumably for the same β-glucan-masking purpose as *H. capsulatum*; however, the role of α-glucan has not been validated in these other dimorphic fungal pathogens.

TLR2, TLR4, Dectin-2, Mincle and the macrophage mannose receptor are pattern recognition receptors for various fungal mannans that potentially recognize mannan-containing PAMPs on the *H. capsulatum* surface. Early studies of *H. capsulatum* binding to human macrophages showed no reduction in yeast binding to macrophages in the presence of excess mannose competitor, suggesting a lack of mannan-type PAMP–pattern recognition receptor interactions [20]. However, recent studies have shown that Dectin-2, but not Mincle, recognizes *H. capsulatum* yeast cell surfaces, and initiates signal transduction in a heterologous signaling system [38]. The functional consequences of Dectin-2 detection of *H. capsulatum* mannans with regard to macrophage responses remain to be determined, as well as the specific mannan-type PAMP recognized.

TLR2 and TLR4 represent two additional signaling receptors that may detect *H. capsulatum* yeasts. TLR signaling is mediated through the MyD88 adaptor protein [46]. Consistent with some recognition of yeasts by TLRs, MyD88-deficient mice show increased susceptibility to *H. capsulatum* infection and show reduced cytokine production, which correlates with a reduced adaptive immune response [37]. At the macrophage level, phagocytes that express TLR2, compared to TLR2-deficient cells, contain more lipid bodies, which correlates with increased leukotriene production [47], suggesting TLR2-based recognition of *H. capsulatum* yeasts occurs. No similar effect was observed in TLR4-deficient cells, indicating the lack of a role for TLR4 recognition of yeasts. A stronger role for TLR2 recognition was found when using cell-wall biochemical fractions [47]. These findings suggest that the cell wall contains PAMPs that are recognized by TLR2, but that the spatial organization of the cell wall in live yeast limits TLR2 recognition by macrophages. Blocking TLR2 or TLR4 does not impair phagocytosis of *H. capsulatum* yeasts [19]; however, yeasts were still able to bind to CR3 in these experiments to mediate uptake.

Cell-surface localized fungal molecules

As the cell wall is the primary surface that interacts with the macrophage, surface-localized factors of *H. capsulatum* yeasts are potential PAMPs that may be recognized or may influence the yeast–macrophage interaction. Cell wall-localized factors have often been investigated due to their role as immunologically dominant epitopes or vaccine substrates. These include the aforementioned Hsp60 protein, the extracellular catalase CatB/M-antigen [48], histone 2B [49] and Hsp70 [50] (Fig. 2). The yeast phase-specific protein Yps3, produced by some lineages of *H. capsulatum* [51–53], associates with the yeast cell surface via interactions with chitin [54]. Purified recombinant Yps3 was shown to bind to and activate TLR2 in microglial cells, leading to TLR2-dependent NF-κB stimulation and chemokine production [55]; however, no interaction of Yps3 in the normal context of a yeast cell was determined, and nor were the consequences of TLR2-dependent yeast interaction with macrophages. While pulmonary infection by *H. capsulatum* yeasts is not influenced by Yps3, *H. capsulatum* yeasts lacking Yps3 show reduced dissemination [56]. A speculative model based on these data suggests that Yps3-TLR2 interactions increase chemokine

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production, resulting in enhanced recruitment of phagocytes that serve as host cells for the yeast and vehicles for extra-pulmonary spread.

Intracellular challenges

Reactive oxygen species

The production of reactive oxygen species (ROS) is a central mechanism used by phagocytes to effect killing of fungal cells (Fig. 3). Phagocytic cells, particularly polymorphonuclear leukocytes and macrophages, produce reactive superoxide through assembly of the NADPH oxidase complex at the plasma or phagosomal membrane. Superoxide (O_2^-), and other reactive oxygen molecules derived from it [e.g. peroxide (H_2O_2) and hydroxyl radicals (•OH)] directly damage macromolecules, resulting in death of the microbe. Phagocyte-generated ROS differ from ROS generated by yeasts as by-products of aerobic respiration in that phagocyte-produced ROS are located extracellularly to the fungal cell. While most organisms possess various intracellular antioxidant enzymes to cope with metabolic-derived ROS, these enzymes are not positioned appropriately to deal with exogenous ROS, particularly superoxide, which does not cross membranes efficiently due to its charged nature. Thus, to survive, successful pathogens that interact with macrophages, and especially those that invade macrophages, must have mechanisms to avoid or rapidly neutralize any phagocyte-derived ROS. The ability to survive challenge with ROS-producing phagocytes specifically characterizes *H. capsulatum* yeast and not mycelia [11], consistent with the separation of the pathogenic and saprobic lifestyles of the two *H. capsulatum* forms.

Histoplasma capsulatum yeasts combat toxic ROS produced by phagocytes by expression of an efficient extracellular superoxide dismutase (Sod3) and catalase (CatB) (Fig. 3). Both *H. capsulatum* Sod3 and CatB are expressed specifically by *H. capsulatum* yeasts but not mycelia, indicating roles adapted to the pathogenic lifestyle of yeasts [57]. *H. capsulatum* Sod3 and CatB enzymes are secreted and associate with the yeast cell wall, positioning them to deal specifically with phagocyte-produced ROS but not yeast cytosolic or mitochondrial ROS [58,59]. Biochemically, extracellular Sod enzymes differ from intracellular Sod enzymes by functioning as monomers and lacking an electrostatic loop domain that guides superoxide to the catalytic metal co-factor [60]. As a consequence, the catalytic site is more accessible, resulting in rapid catalytic rates. *H. capsulatum* yeasts lacking Sod3 function are efficiently killed by phagocytes, and this killing is mediated by phagocyte-produced ROS [58]. *H. capsulatum*'s intracellular superoxide dismutase (Sod1) is unable to protect yeasts from exogenous superoxide or ROS-dependent killing by phagocytes, indicating spatial specificity of Sod3 and Sod1 for phagocyte-produced and intracellular ROS, respectively. Notably, Sod3 protects *H. capsulatum* yeasts from levels of superoxide produced by phagocytes that are sufficient to kill opportunistic fungal pathogens. *In vivo, H. capsulatum* yeasts lacking Sod3 function have attenuated virulence, and this attenuation is dependent on the ability of the host to produce superoxide, confirming the essential role of Sod3 in protecting *H. capsulatum* yeasts from phagocyte-derived ROS. Early studies of the yeast–macrophage interaction appeared to indicate that macrophages fail to produce ROS when stimulated with *H. capsulatum* yeasts [61–63], but no mechanism for how *H. capsulatum* may inhibit ROS generation was determined. However, the identification and analysis of

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Sod3 demonstrates that the lack of detectable oxidative burst in these studies results from highly efficient destruction of ROS by *H. capsulatum* yeasts [58].

Like Sod3, *H. capsulatum*'s CatB catalase is located extracellularly to the yeast cell in order to counter exogenous peroxide stress [59]. Loss of CatB reduced the ability of yeasts to eliminate extracellular peroxide, and slightly reduced survival against phagocytes [59]. Additional loss of the intracellular catalase (CatP) further reduced yeast virulence *in vitro* and *in vivo* [59]. The partial functional redundancy of CatP for CatB, despite its intracellular localization, probably stems from the ability of peroxide to pass through membranes due to lack of charge, unlike superoxide. Without the CatB/CatP defenses, *H. capsulatum* is killed by phagocyte-derived ROS. Together, these data indicate that *H. capsulatum* yeasts ensure their survival in macrophages through destruction of phagocyte-produced ROS by an extracellular ROS defense system consisting of Sod3 and CatB (Fig. 3).

Reactive nitrogen species

In addition to ROS production, activated phagocytes combat fungi by production of reactive nitrogen species (RNS). These species include nitric oxide (NO•), peroxynitrite ($ONOO^-$) and nitrogen dioxide ($•NO_2$). Inhibition of RNS production by phagocytes decreases their ability to control *H. capsulatum* [64,65], suggesting that *H. capsulatum* yeasts are susceptible to RNS. However, RNS is only fungistatic to *H. capsulatum* [65], indicating that *H. capsulatum* yeasts may contend with the lethal, but not the inhibitory, effects of RNS. The mechanism that underlies this ability is unknown, but transcriptional analysis of RNS-treated yeasts identified 59 genes that are up-regulated in response to nitrosative stress [66]. Cytokine activation of phagocytes stimulates RNS production, and reduced pro-inflammatory cytokine levels are correlated with increased susceptibility of mice to *H. capsulatum* infection [64,65,67]. These data suggest that RNS control *H. capsulatum* yeast, but that RNS-based control functions during the adaptive immunity stage of infection rather than the initial infection of phagocytes.

Phagosome acidification

Acidification of the phagosome/phagolysosome contributes to killing of microorganisms by activation of acidic lysosomal hydrolases. Conflicting reports exist regarding whether phagosome–lysosome fusion occurs in the *H. capsulatum*-infected macrophage, depending on the cell type and assay used [68–71]. Regardless, the lumen of the *H. capsulatum*-containing compartment does not become highly acidified. Live *H. capsulatum* yeasts, but not heat-killed yeasts, maintain a relatively neutral pH (~ pH 6.5) in the phagosome/phagolysosome [69,71,72]. Maintenance of this more neutral phagosomal pH is essential for *H. capsulatum* infection of macrophages, as demonstrated by lack of survival of *H. capsulatum* yeast when the intracellular compartment is acidified [73]. A mutation in the 3-hydroxy-3-methyl-glutaryl CoA lyase gene (*HCL1*) results in acidification of the surrounding growth environment due to accumulation and secretion of acidic intermediates of leucine catabolism. This acidification does not impair *in vitro* growth, but severely compromises survival in macrophages, which is correlated with an acidified phagosomal pH [73]. In this study, phagosomal acidification was not dependent on the vacuolar ATPase, indicating that the mutant itself, not the macrophage, artificially acidified the compartment.

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While these data do not indicate that Hcl1 is part of *H. capsulatum*'s mechanism for neutralizing the phagosomal pH, they do provide genetic evidence that *H. capsulatum* must prevent acidification in order to survive in macrophages. The mechanisms behind this feature of *H. capsulatum* intracellular pathogenesis remain unknown.

Nutrient limitation

Intracellular pathogens must not only survive the defenses of the macrophage but also acquire resources for proliferation, and intracellular residence imposes nutritional challenges on *H. capsulatum*. In contrast to growth in rich media *in vitro*, the phagosomal compartment is generally considered to be nutrient-poor [74–77]. This is supported by studies of the intracellular lifestyle of bacteria and fungi, which show major transcriptional changes of nutritional genes in organisms within the phagosome compared to in culture [78–81]. In addition, removal of genes involved in nutrient synthesis or utilization may affect virulence without affecting *in vitro* growth of pathogenic bacteria and fungi [82–86]. To proliferate in macrophages, *H. capsulatum* yeast must catabolize whatever carbon, nitrogen and sulfur sources are available, as well as assimilating trace elements essential for life processes. Where nutrient gaps exist in the intracellular compartment, intracellular pathogens must possess sufficient biosynthetic capabilities to satisfy metabolic demands. Natural auxotrophies and derived auxotrophic *H. capsulatum* strains are enabling determination of which nutrient sources are and are not available, and which may be utilized in the intracellular compartment for yeast proliferation.

Limitation of essential metals

Biologically available iron is rare in the environment, and, within the macrophage, phagosome acquisition of iron is an even greater challenge. *H. capsulatum* yeasts have multiple strategies to acquire iron within host cells. Although *H. capsulatum* yeasts prevent acidification of the phagosomal compartment, the pH must still be lower than pH 7 in order to liberate iron from transferrin [87]. Thus, *H. capsulatum* must balance the need to avoid activation of acidic hydrolases of the macrophage with the need to acidify the compartment sufficiently to obtain iron. This balance is achieved by maintaining the pH at ~ 6.0–6.5, which is acidic enough to release one of the two iron atoms from transferrin but not sufficiently acidic for hydrolase activation. Consistent with this, chloroquine, which raises the phagosomal pH, decreases *H. capsulatum* survival in macrophages by reducing available intracellular iron [87].

A second method by which *H. capsulatum* acquires limited iron intracellularly is by secretion of iron-chelating siderophores (Fig. 4). *H. capsulatum* yeasts produce hydroxamate siderophores that scavenge iron from transferrin [88]. Iron limitation induces both transcriptional- and protein-level changes in *H. capsulatum* yeasts [89,90], including Sid1, a key enzyme in siderophore biosynthesis. Transcriptional changes are mediated by the iron-responsive GATA family transcription factor Sre1 [89]. Mutants of *H. capsulatum* that are unable to make siderophores have reduced proliferation in cultured macrophages [89,91]. *In vivo*, loss of siderophore production in North American type 2 strains drastically reduces pulmonary infection [91] at early stages. In contrast, a Panama strain that is deficient for siderophore synthesis did not show attenuation until day 15 after infection, a time point that

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coincides with adaptive immunity [89]. This discrepancy suggests that Panama strains have alternative iron acquisition strategies that operate in addition to siderophores.

Histoplasma capsulatum yeasts utilize ferrous iron, and thus express multiple iron-reducing systems and iron transporters. Biochemical studies of extracellular iron reduction demonstrated that *H. capsulatum* yeasts produce three reductases, two soluble and one cell-associated [92,93]. Follow-up work identified one of these as a γ-glutamyl transferase (Fig. 4), which catalyzes a two-step reduction of ferric iron using reduced glutathione [94]. *H. capsulatum* yeasts lacking γ-glutamyl transferase function are impaired in the ability to proliferate within cultured macrophages, confirming acquisition of iron is a challenge faced by *H. capsulatum*. In addition, some strains (e.g. those of the Panama lineage) have genes encoding a high-affinity iron transport system (Fet3 and Ftr1) [91]. *Paracoccidioides* species and *B. dermatitidis* also produce hydroxymate siderophores and ferric reductases similar to those of *H. capsulatum* [95–97], suggesting common strategies for iron acquisition and utilization. However, the necessity for siderophores for fungal virulence in these organisms has not yet been established.

Intracellular nutrient availability is dynamic, as host responses to infection may cause limitation of certain nutrients, a concept termed 'nutritional immunity' [98]. Cytokine activation of macrophages may cause a further reduction in the free iron available to *H. capsulatum* yeasts in the phagosome, which provides one explanation of why Panama strains lacking siderophores are not impaired until adaptive immunity activates the macrophages [89]. Host cells actively remove iron when activated with interferon γ, which stimulates the sequestration of iron in an attempt to inhibit growth of the pathogen [99,100]. Zinc is another element that is central to the function of many metalloenzymes, and zinc availability is modulated by cytokine activation of macrophages. Treatment of *H. capsulatum*-infected macrophages with granulocyte-macrophage colony-stimulating factor decreased cellular levels of available iron and zinc [101]. This is correlated with the ability of granulocyte-macrophage colony-stimulating factor-treated macrophages to inhibit *H. capsulatum* growth and replication and to increase macrophage ROS production and phagosomal acidification [102]. In non-activated macrophages, *H. capsulatum* may effectively battle the host for iron and zinc, but activation of macrophages may restrict the availability of elements necessary for intracellular yeast proliferation (Fig. 4).

Pyrimidine limitation

The *H. capsulatum*-containing phagosomal compartment lacks available nucleic acids for *H. capsulatum* utilization. This was demonstrated by the isolation and characterization of a *H. capsulatum* uracil auxotroph. Mutants lacking Ura5 function grow *in vitro* as long as the medium is supplemented with uracil, indicating that *H. capsulatum* yeasts possess pyrimidine transport functions [103]. However, the uracil auxotroph is unable to grow in macrophages in culture, and lacks the ability to proliferate *in vivo* [104]. These results indicate that *H. capsulatum*'s pyrimidine biosynthetic pathway is essential for overcoming the lack of available uracil in the macrophage phagosome.

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Scarcity of essential vitamins

To proliferate intracellularly, *H. capsulatum* yeasts use *de novo* biosynthesis of essential vitamins to overcome their absence in the phagosome. Loss of function of Rib2 (diaminohydroxyphosphoribosylaminopyrimidine deaminase) creates a riboflavin auxotrophy that may be rescued *in vitro* by supplementation with riboflavin [105]. However, there is essentially no replication of the *rib2* mutant in cultured macrophages, and the mutant is severely attenuated *in vivo*. The intracellular yeasts are not killed during initial infection but are unable to proliferate intracellularly. Similar infection-related yeast growth inhibition results if *H. capsulatum* pantothenate biosynthesis is blocked, but not when biotin synthesis is impaired [105]. *In vitro*, wild-type *H. capsulatum* yeasts are able to synthesize all essential vitamins except for thiamine [105], which enables them to overcome the lack of riboflavin and pantothenate in the phagosome (Fig. 4). The growth of the biotin auxotroph in macrophages suggests that the phagosome contains sufficient biotin or that this co-factor is efficiently scavenged by intracellular yeasts.

Organic sulfur

Interestingly, *H. capsulatum* yeasts, but not mycelia, are auxotrophic for cysteine. This has been attributed to temperature-dependent lack of sulfite reductase expression, thereby preventing yeasts from incorporating inorganic sulfate into cysteine [106–109]. Despite the need for organic sulfur for yeast growth *in vitro*, wild-type *H. capsulatum* yeasts are able to proliferate within macrophages and establish infection *in vivo*, suggesting that the intracellular environment has ample organic sulfur to support *H. capsulatum* growth [110]. In addition, an undefined cysteine auxotroph strain derived by mutagenesis is fully virulent *in vivo* [111,112]. These results suggest that, while pyrimidines and essential vitamins are limiting in the macrophage, necessitating *de novo* synthesis by *H. capsulatum* yeasts, cysteine or an organic sulfur source is readily available to intracellular *H. capsulatum* yeasts.

Paracoccidioides brasiliensis shows a similar inorganic sulfur auxotrophy in the yeast phase, as organic sulfur is required for the transition from mycelia to yeasts [113–116] and many sulfur metabolism genes are differentially regulated between the yeast and mycelial phases [116–118]. The similar yeast requirement for organic sulfur in *H. capsulatum* and *P. brasiliensis* indicates that sufficient organic sulfur is available intracellularly [119]. Up-regulating the inorganic sulfur assimilatory pathway (by knockdown of the negative regulator Sulfur control protein) in *P. brasiliensis* rescues the dependence on organic sulfur for mycelial to yeast transition; however, the resulting ATP and redox imbalance impairs yeast growth [115]. These data for *H. capsulatum* and *P. brasiliensis* suggest that yeasts, but not mycelia, have streamlined expression of sulfur metabolism genes to exploit available organic sulfur in the phagosome. Interestingly, *Legionella pneumophila*, a bacterial pathogen that resides within the phagosome, is also naturally auxotrophic for cysteine [120]. It remains unknown what the actual organic sulfur compound(s) is (are) that are utilized by intracellular pathogens.

Undefined obstacles to intracellular proliferation

The first virulence factor identified for *H. capsulatum* was the small secreted calcium-binding protein Cbp1 [121]. Cbp1 has nanomolar affinity for calcium [122,123], but it

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remains unclear what role calcium may play in Cbp1 function or whether Cbp1 contributes to calcium homeostasis of intracellular yeasts. Cbp1 is abundantly produced specifically by *H. capsulatum* yeasts (not by mycelia), consistent with its function in *H. capsulatum* pathogenesis, and the protein is secreted into the phagosomal lumen [124]. The compact structure of Cbp1, created by intramolecular disulfide bonds, makes Cbp1 resistant to proteases that may be encountered in a phagolysosome. Despite the molecular and structural information on Cbp1, how this secreted protein contributes to *H. capsulatum* pathogenesis remains unknown, and consequently the obstacles that Cbp1 enables intracellular *H. capsulatum* yeast to overcome are undefined.

Conclusions

Histoplasma capsulatum is one of only a few microbial pathogens with a nearly exclusive intracellular lifestyle. Even though many human pathogenic fungi can exist within macrophages, they often have strategies for escape from the host cell [125–127]. In contrast, *H. capsulatum* tends to remain inside the macrophage for an extended time, proliferating to high numbers intracellularly. While opportunistic fungal pathogens are readily controlled by cells of the innate immune system, *H. capsulatum*, as a primary pathogen, survives and replicates within host phagocytes.

Histoplasma capsulatum's success in establishing a permissive intracellular environment depends on multiple mechanisms to exploit the macrophage as its host cell. Two major obstacles facing intracellular pathogens are how to enter host cells without being recognized as a microbial invader and how to survive the onslaught of antimicrobial defense molecules (e.g. ROS and phagosome acidification). For the first task, *H. capsulatum* promotes its uptake by macrophages by interacting with phagocytic receptors while simultaneously minimizing recognition by signaling receptors. It achieves this by masking immunostimulatory molecules. *H. capsulatum* yeasts also effectively resist killing by macrophages by production of secreted defenses against fungicidal ROS and modulation of the phagosomal environment to prevent activation of lysosomal hydrolases. Production of external factors to specifically eliminate phagocyte-derived ROS is a common characteristic of pathogens that are able to infect and survive within phagocytes [128–132]. Thus, *H. capsulatum* accomplishes both stealth and survival, thereby preventing elimination and control by its host cell.

Successful intracellular pathogens need to do more than simply survive entry into phagocytes. Once established as an intracellular resident, *H. capsulatum* yeasts successfully acquire the nutrients necessary for proliferation. At least three mechanisms operate to capture limited iron, highlighting the challenge of nutritional immunity facing intracellular yeasts. Nevertheless, acquisition of essential metals may be *H. capsulatum*'s Achilles heel, as indicated by improved phagocyte control of *H. capsulatum* yeasts when macrophages are activated and metal sequestration is enhanced [87,99,102,133]. Although the primary carbon, nitrogen and sulfur sources utilized by *H. capsulatum* yeasts remain undefined, *H. capsulatum* yeasts have sufficient *de novo* synthesis pathways for biological molecules to provide for growth and replication, including biosynthesis of pyrimidines and essential vitamins. On the other hand, some nutrients, such as organic sulfur, appear to be sufficiently

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available in the phagosome to allow the proliferation of yeasts that are dependent on organic sulfur. These capabilities enable *H. capsulatum* to convert a challenging nutrient-poor phagosome into a permissive intracellular niche. Thus, a combination of survival strategies and nutrient acquisition mechanisms facilitate *H. capsulatum*'s success as an intracellular pathogen of macrophages.

Abbreviations

Ags1	α-(1,3)-glucan synthase
Amy1	α-amylase-like protein
Cat	catalase
Cbp1	calcium-binding protein
PAMP	pathogen-associated molecular pattern
ROS	reactive oxygen species
Sod	superoxide dismutase
SP	surfactant protein
TLR	Toll-like receptor

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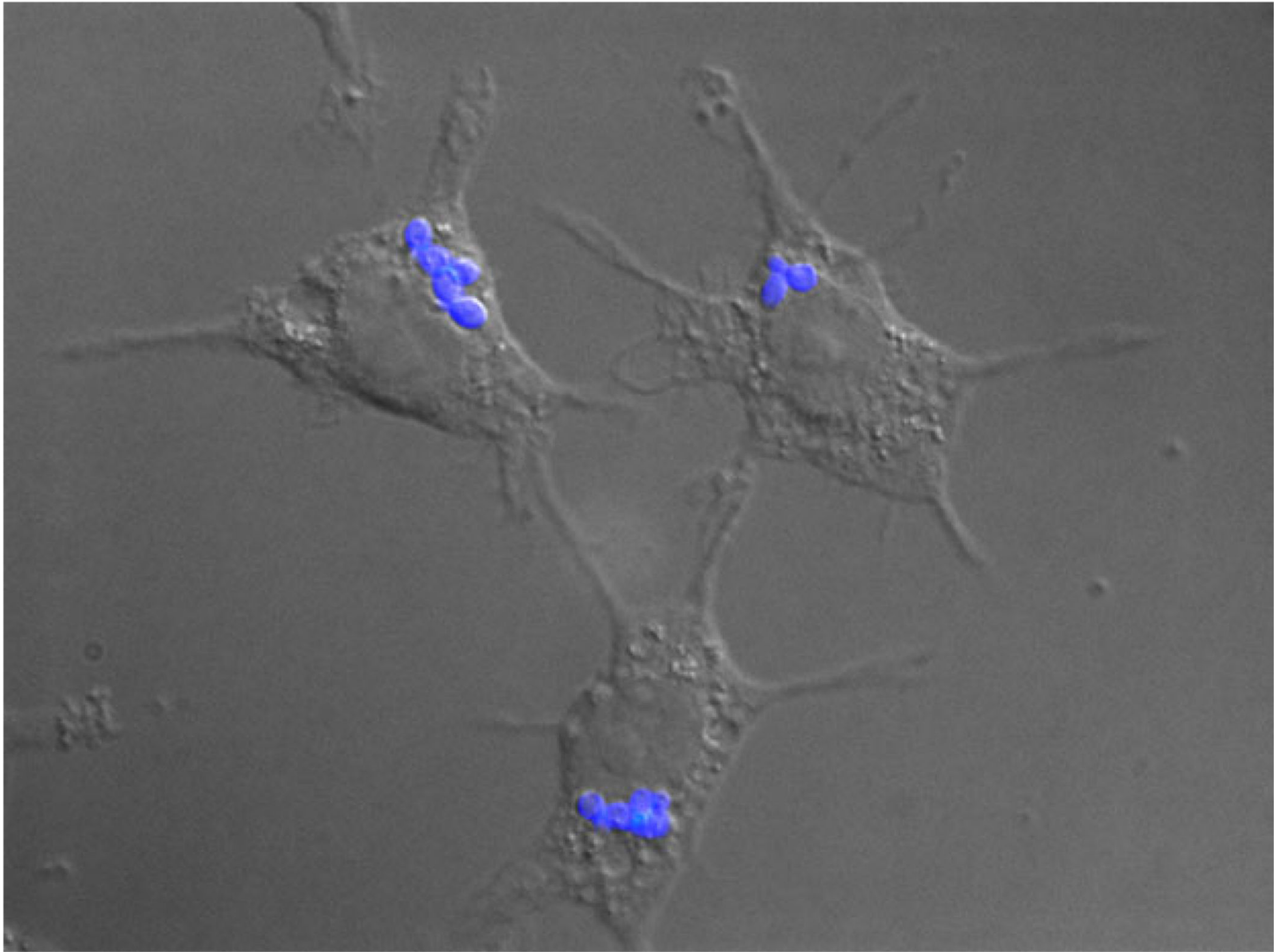

Fig. 1.
Histoplasma capsulatum is an intracellular pathogen of macrophages. The image shows *H. capsulatum* yeasts (blue fluorescence) within phagocytic P388D1 macrophage cells. Samples were fixed 2 h post-infection, and yeasts are stained using Uvitex (Polysciences, Inc., Warrington, PA).

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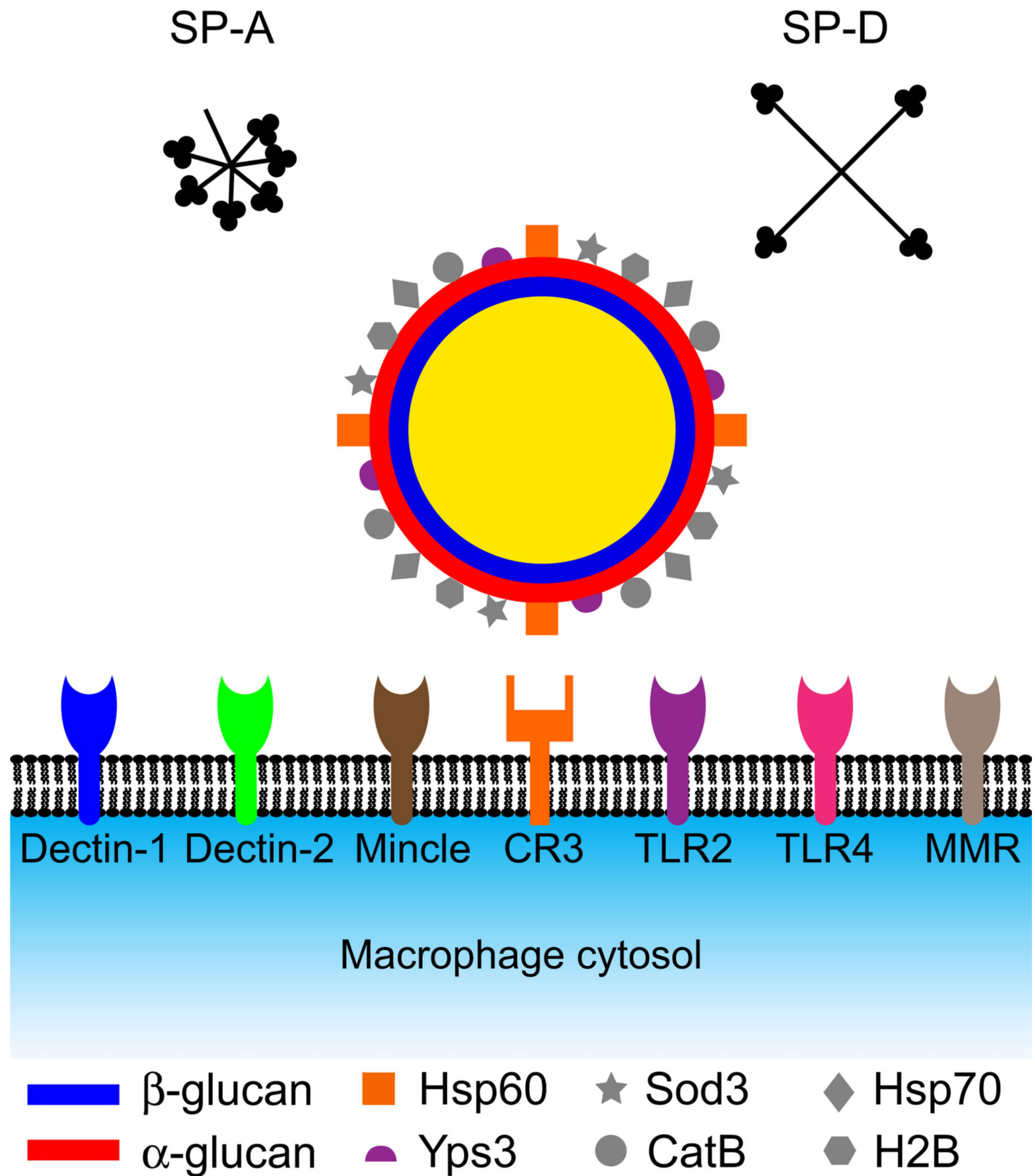

Fig. 2.

Subversion of extracellular obstacles to infection by *Histoplasma capsulatum*. Within alveolar spaces, *H. capsulatum* encounters the antifungal surfactant collectins SP-A and SP-D. To gain entry into macrophages for refuge from surfactant proteins, *H. capsulatum* yeasts interact with the phagocytic receptor CR3 (orange receptor with square binding pocket) using Hsp60 (orange squares) as the ligand. A number of signaling receptors (round binding pockets) may potentially interact with the yeast cell surface. The α-glucan polysaccharide layer (red) of the *H. capsulatum* cell wall conceals the immunostimulatory β-glucan layer

(blue), preventing recognition of yeasts by the phagocyte β-glucan receptor Dectin-1 (blue receptor). Other cell-wall proteins that potentially interact with the macrophage include Yps3 (purple semi-circles), which may interact with TLR2 receptors, Hsp70 (diamonds), Sod3 (stars), CatB (circles) and H2B (hexagons). Mannose-type receptors on the macrophage include Dectin-2, Mincle, TLR2, TLR4 and the macrophage mannose receptor (MMR).

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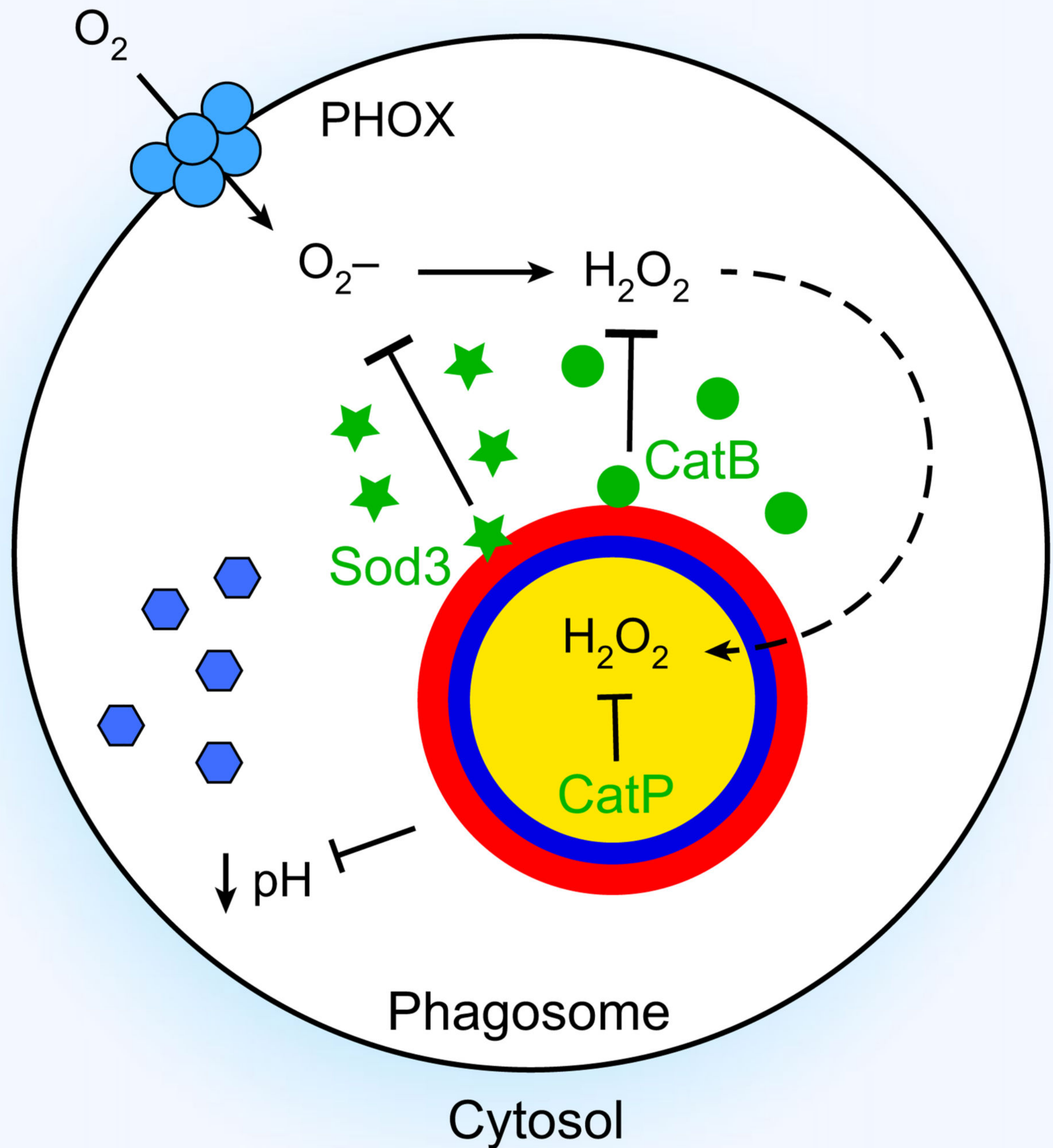

Fig. 3.

Histoplasma capsulatum mechanisms for survival in phagocytes. The schematic depicts a *H. capsulatum* yeast cell within a macrophage phagosome. The phagocyte NADPH oxidase complex (PHOX; blue membrane complex) produces superoxide (O_2–) and peroxide (H_2O_2) within the phagosome. *H. capsulatum* eliminates these reactive oxygen molecules through production of extracellular superoxide dismutase Sod3 (green stars) and extracellular catalase CatB (green circles). If any H_2O_2 escapes destruction by CatB and enters the yeast cell (dashed arrow), further protection is provided by intracellular CatP. Acidification of the

phagosome lumen presents an additional obstacle to intracellular survival by activation of acidic hydrolases (blue hexagons). *H. capsulatum* yeasts block acidification by an unknown mechanism, to maintain a near neutral pH.

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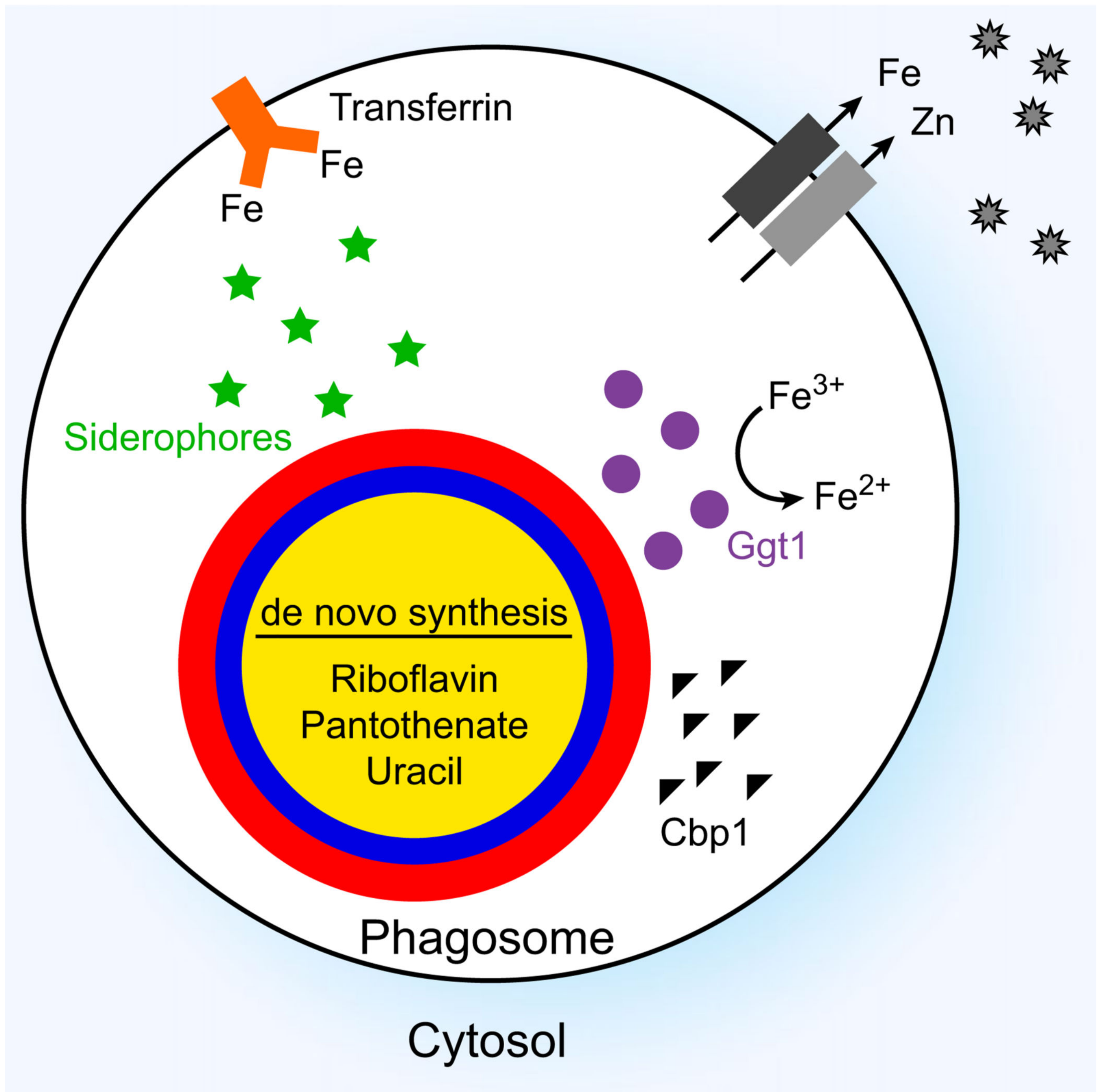

Fig. 4.

Histoplasma capsulatum mechanisms to cope with nutritional limitations and other obstacles to intracellular proliferation. *H. capsulatum* may acquire iron from transferrin (orange). Phagocytes restrict the availability of essential metals such as iron and zinc through membrane transporters (gray) and sequestering metallothioneins (gray star). *H. capsulatum* yeasts produce siderophores (green stars) with high affinity for iron to acquire iron within the phagosome. In addition, yeast cells produce γ-glutamyl transferase (Ggt1; purple circles) to reduce iron within the phagosome. To overcome nutrient limitations, *H. capsulatum* yeasts

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produce compounds such as uracil and the essential vitamins riboflavin and pantothenate using de novo synthesis pathways. The calcium-binding protein Cbp1 (black triangles) is highly expressed by yeasts, which facilitates intracellular pathogenesis through an unknown mechanism.