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Evolutionary aspects of urea utilization by fungi

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Abstract

The higher fungi exhibit a dichotomy with regard to urea utilization. The hemiascomycetes use urea amidolyase (DUR1,2) whereas all other higher fungi use the nickel-containing urease. Urea amidolyase is an energy dependent biotin-containing enzyme. It likely arose prior to the Euascomycete/Hemiascomycete divergence ca. 350 million years ago by insertion of an unknown gene into one copy of a duplicated methylcrotonyl CoA carboxylase (*MccA*). The dichotomy between urease and urea amidolyase coincides precisely with that for the Ni/Co transporter (Nic1p) which is present in the higher fungi that use urease and absent in those that do not. We suggest that the selective advantage for urea amidolyase is that it allowed the hemiascomycetes to jettison all Ni^{2+} and Co^{2+} dependent metabolism and thus to have two fewer transition metals whose concentrations need to be regulated. Also, the absence of *MccA* in the hemiascomycetes coincides with and may explain their production of fusel alcohols.

Introduction

We have long been interested in the role of nitrogen sources in regulating fungal dimorphism. For instance, the growth morphology of *Ceratocystis ulmi* and *Trigonopsis variabilis* could be modulated by the source of nitrogen. For *C. ulmi*, the cells grew as yeasts with proline and as hyphae with ammonia, arginine, and most other nitrogen sources (Kulkarni and Nickerson, 1981) while for *T. variabilis*, the cells grew as budding yeasts with ammonium sulfate and as triangles with methionine (Sentheshanmuganathan and Nickerson, 1962). One nitrogen source that has been understudied in *C. albicans* is urea. This inattention likely derives from numerous reports that *C. albicans* lacks urease (Odds, 1988) even though Dastidar et al (1967) reported that most strains of *C. albicans* grew well with urea as the sole source of nitrogen. A partial resolution of this impasse derives from the fact that *C. albicans* uses urea amidolyase to hydrolyze urea (Ghosh et al, 2009). The enzyme urea amidolyase, encoded by *DUR 1, 2* (Degradation of URea), was first characterized in the yeast *Candida utilis* (Roon and Levenberg, 1972). This cytoplasmic, biotin-dependent enzyme (Roon et al, 1972) consists of a single protein chain with domains for both urea carboxylase and allophanate hydrolase activity (Cooper et al, 1980).

i. urea $\rightarrow 2NH_3 + CO_2$ urease

ii. urea + ATP + $HCO_3^- \rightarrow$ allophanate + ADP + P_i urea carboxylase

iii. allophanate $\rightarrow 2NH_3 + 2CO_2$ allophanate hydrolase

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In *Saccharomyces cerevisiae* the actual inducer for *DUR1,2* is allophanate, also known as urea carboxylate, rather than urea itself (Cooper, 1982). Allophanate is the chemical intermediate for the multifunctional Dur1, 2p, see equations (ii) and (iii). Here we report the conservation of urea utilization in fungi and the phylogenetic distribution of urease and urea amidolyase in the fungi.

Distribution of Urease and Urea Amidolyase in the Fungi

To identify which fungi have urease and which have urea amidolyase, we examined 22 available fungal genomes spanning the Ascomycetes and Basidiomycetes for the presence of the respective genes. There is a dichotomy. Urease (Fig. 1A in red) was found in all of the fungi except for members of the hemiascomycetes (Fig. 1A in black). The hemiascomycetes are those Ascomycetes which do not form fruiting bodies. The results are consistent with loss of the urease gene sometime prior to the Euascomycete – Hemiascomycete divergence ca. 350 million years ago (Galagan et al, 2005).

The dual function urea amidolyase Dur 1, 2 comes in two sizes: the longer of ca. 1800 aa (Fig. 1B in red) has an ca. 600 aa “amidase” domain fused at the N-terminus, whereas the shorter of ca. 1200 aa (Fig. 1B in orange) has not. The longer *DUR 1, 2* is present in all hemiascomycetes examined, including *C. albicans*, whereas both the longer and shorter versions are found in subsets of the euascomycetes (Fig. 1B). One explanation is that *DUR 1, 2* arose via duplication of the gene for MccA, mitochondrial biotin-containing methylcrotonyl-CoA carboxylase (ca. 700 aa), followed by fusion of one of the MccA genes with another still unidentified gene (ca. 500 aa) to create *DUR1,2*. In the process, the allophanate hydrolyase domain (Dur 2) was inserted in the biotin-containing MccA. The Zygomycetes and Basidomycetes have only MccA (Fig. 1B in green), while the Hemiascomycetes are missing MccA (Fig. 1B). Split red-green and orange-green labels (Fig. 1B) indicate euascomycete species that contain both *DUR1,2* and *MccA*. *Schizosaccharomyces pombe* does not contain either *DUR1,2* or *MccA*. *DUR1,2* likely originated prior to the split between the euascomycetes and hemiascomycetes and was subsequently lost from several euascomycete lineages, although the possibility for multiple independent origins cannot be eliminated.

There is ample precedent for metabolic dichotomies in the fungi. For instance, in lysine biosynthesis (Vogel, 1965) the two pathways for lysine biosynthesis are named after the intermediates that are characteristic of the paths, α-aminoadipic acid (AAA) and diaminopimelic acid (DAP). Euglenaceae and all of the fungi use the AAA pathway, whereas all other lysine prototrophs use the DAP pathway. No intermediates or enzymes are common to the two pathways (Vogel, 1965).

The distinct phylogenetic trees for urease (Fig. 1A) and urea amidolyase (Fig. 1B) raise three further questions regarding how and why those changes occurred. The first concerns why two plant pathogens, *Gibberella zeae* and *Magnaportha grisea*, retain both urease and urea amidolyase when plants recycle virtually all of their amino groups and thus do not excrete urea. The second concerns the energetics of biotinylated enzymes. Most eukaryotes have only four biotin-containing enzymes: pyruvate carboxylase, propionyl-CoA carboxylase, acetyl-CoA carboxylase, and methylcrotonyl-CoA carboxylase (Samols et al, 1988). Why do the hemiascomycetes use an energy-dependent, biotin-containing urea amidolyase system when the same overall reaction could be accomplished by the simpler urease? This question becomes even more germane when we consider that all strains of *C. albicans* are biotin auxotrophs (Odds, 1988), and it has long been known that 2 to 4 times as much biotin is required for maximum growth of *S. cerevisiae* on urea, allantoic acid, or allantoin as sole nitrogen sources (DiCarlo et al, 1953).

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Loss of Ni^{2+}/Co^{2+} enzymes

Our current thinking is that *DUR1,2* allows the hemiascomycetes to retain urea as a nitrogen source while jettisoning their last Ni^{2+} -containing enzyme. As part of a comparative genomic analysis, Zhang et al (2009) examined sixty three fungal genomes for consensus/predictive sequences associated with Ni^{2+}/Co^{2+} transporters and the use of nickel and cobalt by fungi. Among biometals, nickel and cobalt are considered together because they are used at particularly low levels and often share a transport system. For the cobalt-containing vitamin B_{12}, three coenzyme B_{12}-dependent enzymes: methionine synthase, methylmalonyl-CoA mutase, and the B_{12}-dependent ribonucleotide reductase, were not found in any of the 63 fungal genomes (Zhang et al, 2009). Thus, *in silico* analysis concluded that the higher fungi as a group do not use Co^{2+} or any coenzyme B_{12}-dependent enzymes. However, marine lower fungi belonging to the Phycomycetes may still have a functional requirement for cyanocobalamin and exhibit B_{12} deficiencies (Goldstein and Belsky, 1963).

The situation for nickel was more intriguing in that there was a precise dichotomy between the hemiascomycetes and the rest of the higher fungi. Zhang et al, (2009) screened for the nickel/cobalt transporter (Nic1p) and the Ni-dependent enzyme urease. Neither was present in any of the 24 hemiascomycete genomes examined, but both genes were present in all eight of the Basidiomycetes, both of the Schizosaccharomycetes, and 28 of the 29 Euascomycetes (Zhang et al, 2009). The exception was *Aspergillus terreus* ATTC 20542. Urease was the only Ni-dependent protein identified, and the taxonomic distribution of the nickel transporter and urease coincided exactly with that shown in Fig. 1A.

Thus, the hemiascomycetes do not have any Ni or Co dependent enzymes, thus avoiding the delicate balance of acquiring the necessary trace levels of Ni^{2+} and Co^{2+} without exceeding the threshold levels at which those transition metals become toxic. For instance, Mackay and Pateman (1980) described a mutant of *A. nidulans* for which, with urea as the sole nitrogen source, 0.1 mM Ni^{2+} was required but 1mM Ni^{2+} was toxic. By using *DUR1,2* instead of urease, the Hemiascomycetes can eliminate Ni^{2+}/Co^{2+} transporters and have two less essential transition metals. Also, humans do not utilize nickel for major metabolic processes, and nickel is generally viewed as a toxic or carcinogenic metal (Dosanjh and Michel, 2006). Switching to urea amidolyase would allow hemiascomycetes such as *C. albicans* to achieve urea degradation and kidney colonization in a nickel deficient human host.

Fusel Alcohols

The hemiascomycetes as a group have replaced methylcrotonyl-CoA carboxylase with urea amidolyase. Are there any phenotypes or negative consequences associated with this swap? MccA (EC 6.4.1.4) catalyzes the ATP-dependent carboxylation of 3-methylcrotonyl CoA from 3-methylglutaconyl CoA. It is certainly involved in leucine catabolism (Rodriguez et al (2004) and the production of fusel alcohols by yeasts may be a consequence of the loss of MccA. Fusel alcohols are derived from amino acid catabolism via a pathway proposed by Ehrlich (1907). For amino acids assimilated by the Ehrlich pathway (valine, leucine, isoleucine, methionine, and phenylalanine), after the initial transamination reaction the resulting α – κeto acids cannot be redirected into central metabolism and are instead decarboxylated, reduced, and excreted (Hazlewood et al, 2008). The suggestion that fusel alcohol production derives from the loss of MccA predicts that only the hemiascomycetes should be capable of fusel alcohol production. This prediction has been partially confirmed by Penalva and associates. They found that Δ*mcc* strains of *Aspergillius nidulans* could not grow on leucine as the sole carbon source and accumulated 3-hydroxyisovaleric acid in the culture supernants. Although MccA is associated primarily with leucine catabolism (Rodriguez et al 2004), we note that Northern analysis of *A. nidulans* mycelia revealed that *MccA* and *MccB* transcription was

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elevated in media containing 30 mM of leucine, valine, isoleucine, methionine, or phenylalanine but not in any of the other four amino acids tested (Rodriquez et al, 2004).

Urea and Pathogenicity

Urea catabolism has relevance because urease is a virulence factor in at least two pathogenic fungi Cryptococcus neoformans (Cox et al, 2000) and Coccidioides immitis (Cole, 1997) and two bacteria *Helicobacter pylori* (Eaton et al, 1991) and *Proteus mirabilis* (Jones et al, 1990). Having cytoplasmic urea catabolism (urea amidolyase) permits urea-dependent signalling pathways related to fungal pathogenicity (Ghosh et al, 2009). We examined the role of arginine-induced germ tube formation in the escape of *C. albicans* from murine macrophages (Ghosh et al, 2009). Our studies link the work of Lorenz et al (2004), who showed that the genes for L-arginine biosynthesis were induced following internalization by macrophages, with that of Sims (1986) and Muhlschlegel's group (Bahn and Mühlschlegel, 2006; Klengel et al, 2005) who showed that elevated CO_2 triggers hyphal growth. We connected these two observations via the enzymes L-arginase (Car1p), which converts arginine to urea, and urea amidolyase (Dur1,2p) which produces CO_2. At that time we created a *dur1,2/dur1,2* mutant from a wild type parent (A72) and then reconstituted it. The dur1,2/dur1,2 mutant was unable to: grow on urea as the sole nitrogen source, stimulate germ tube formation in response to L-arginine or urea, or escape from the murine macrophage cell line RAW 264.7. These abilities were restored in the reconstituted strains (Ghosh et al, 2009). Finally, ongoing studies also show that *DUR1,2* is a virulence factor for *C. albicans* (Navarathna and Roberts, unpublished).

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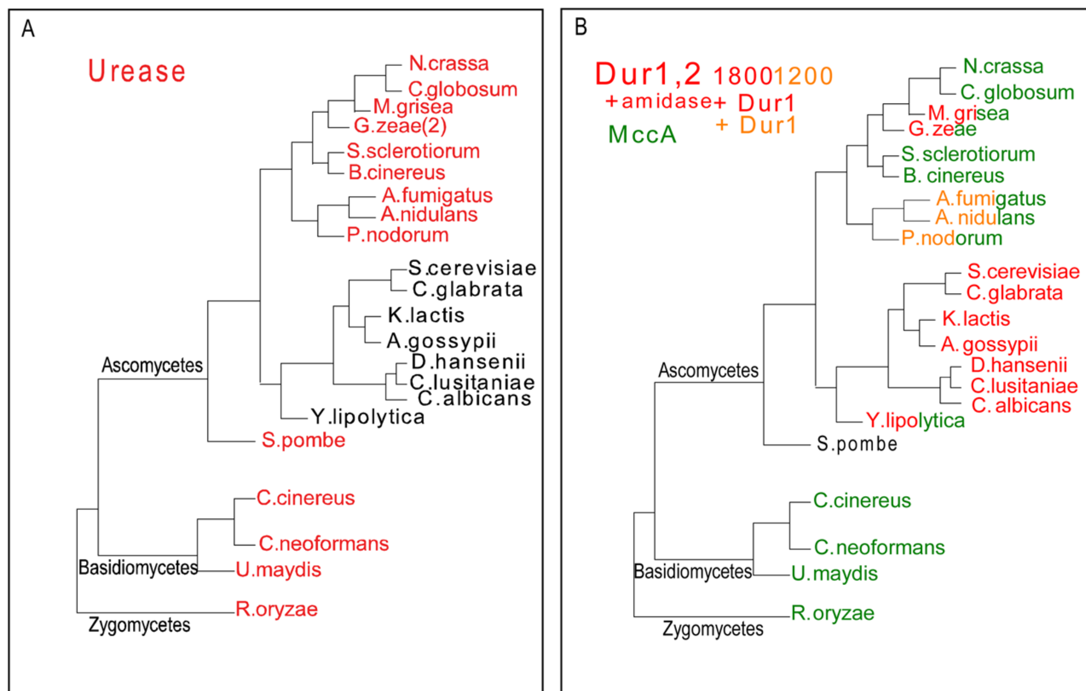

Fig. 1.

Phylogeny of fungal urease, urea amidolyase, (Dur1,2), and methylcrotonyl-CoA carboxylase. Sequences of fungal proteins were obtained from NCBI (www.ncbi.nlm.nih.gov) and the Fungal Genome Initiative (www.broad.mit.edu/annotation/fgi/). All BLAST searches were conducted using default parameters. MacVector software (Oxford Molecular Sciences, Inc., Hunt Valley, MD) was used for processing and analysis of sequences. The dendogram was prepared in PowerPoint and represents the current view of fungal phylogeny as presented by James et al. (2006). **1A.** The presence (red) or absence (black) of urease homologues was identified using *Cryptococcus neoformans* URE1 (AF006062) as a query for BLASTp searches. **1B.** The presence of full-length Dur1,2 (i.e., ~1800 amino acids) is indicated in red, intermediate length Dur1,2 (i.e., ~1200 amino acids) is indicated in orange, and methylcrotonyl-CoA carboxylase is indicated in green. Split red-green and orange-green labels indicate species that contain both Dur1,2 and methylcrotonyl-CoA carboxylase while black indicates that none of the above genes was detected. Dur1, 2 and MccA homologues were identified using *S. cerevisiae* Dur 1, 2 (CAA85172) as a query for BLASTp searches.