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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 before the Euascomycete/Hemiascomycete divergence c. 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 is 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 metabolisms 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 & Nickerson, 1981), while for *T. variabilis*, the cells grew as budding yeasts with ammonium sulfate and as triangles with methionine (Sentheshanmuganathan & Nickerson, 1962). One nitrogen source that has been understudied in *Candida 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 *DUR1,2* (Degradation of URea), was first characterized in the yeast *Candida utilis* (Roon & 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).

(1)	Urea $\rightarrow$ 2NH$_3$+CO$_2$	Urease
(2)	Urea+ATP+HCO$_3^-$ $\rightarrow$ allophanate+ADP+P$_i$	Urea carboxylase
(3)	Allophanate $\rightarrow$ 2NH$_3$+2CO$_2$	Allophanate hydrolase

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 (2) and (3). 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 that do not form fruiting bodies. The results are consistent with loss of the
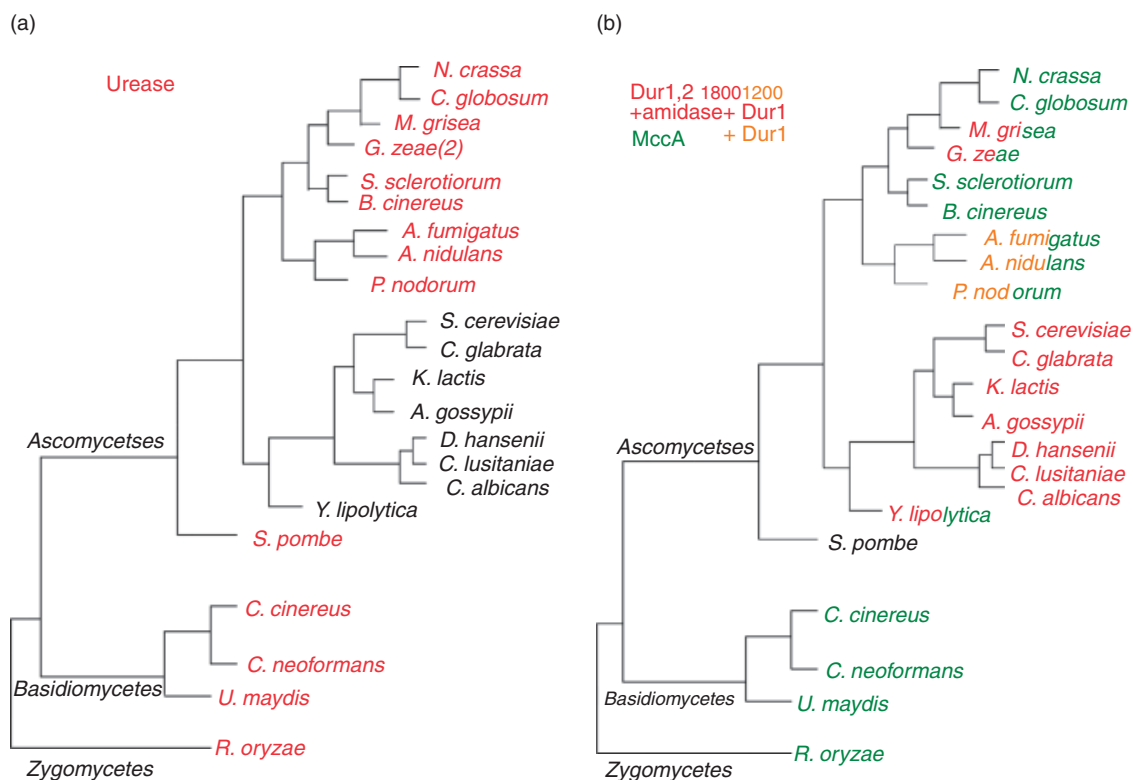
(a)

Urease

N. crassa
C. globosum
M. grisea
G. zeae(2)
S. sclerotiorum
B. cinereus
A. fumigatus
A. nidulans
P. nodorum
S. cerevisiae
C. glabrata
K. lactis
A. gossypii
D. hansenii
C. lusitaniae
C. albicans
Y. lipolytica
Ascomycetses
S. pombe

C. cinereus
C. neoformans
U. maydis
Basidiomycetes
R. oryzae
Zygomycetes

(b)

Dur1,2 1800 1200
+amidase+ Dur1
MccA   + Dur1

N. crassa
C. globosum
M. grisea
G. zeae
S. sclerotiorum
B. cinereus
A. fumigatus
A. nidulans
P. nodorum
S. cerevisiae
C. glabrata
K. lactis
A. gossypii
D. hansenii
C. lusitaniae
C. albicans
Y. lipolytica
Ascomycetses
S. pombe

C. cinereus
C. neoformans
U. maydis
Basidiomycetes
R. oryzae
Zygomycetes

Fig. 1. Phylogeny of fungal urease, urea amidolyase, (Dur1,2), and MccA. Sequences of fungal proteins were obtained from NCBI (http://www.ncbi. nlm.nih.gov) and the Fungal Genome Initiative (http://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). (a) The presence (red) or absence (black) of urease homologues was identified using *Cryptococcus neoformans* URE1 (AF006062) as a query for BLASTP searches. (b) The presence of full-length Dur1,2 (i.e. ~1800 aa) is indicated in red, intermediate-length Dur1,2 (i.e. ~1200 aa) is indicated in orange, and MccA is indicated in green. Split red-green and orange-green labels indicate species that contain both Dur1,2 and MccA, while black indicates that none of the above genes was detected. Dur1, 2 and MccA homologues were identified using *Saccharomyces cerevisiae* Dur 1, 2 (CAA85172) as a query for BLASTP searches.

urease gene sometime before the Euascomycete–Hemiascomycete divergence *c.* 350 million years ago (Galagan *et al.*, 2005).

The dual function urea amidolyase Dur1,2 occurs in two sizes: the longer of *c.* 1800 aa (Fig. 1b in red) has a *c.* 600 aa 'amidase' domain fused at the N-terminus, whereas the shorter of *c.* 1200 aa (Fig. 1b in orange) does not. The longer *DUR1,2* is present in all hemiascomycetes examined, including *C. albicans*, whereas both the longer and the shorter versions are found in subsets of the euascomycetes (Fig. 1b). One explanation is that *DUR1,2* arose via duplication of the gene for methylcrotonyl CoA carboxylase (MccA), mitochondrial biotin-containing MccA (*c.* 700 aa), followed by fusion of one of the *MccA* genes with another still unidentified gene (*c.* 500 aa) to generate *DUR1,2*. In the process, the allophanate hydrolyase domain (Dur2) was inserted into 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 before 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 MccA (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 two to four times as much biotin is required for the maximum growth of *S. cerevisiae* on urea, allantoic acid, or allantoin as the sole nitrogen sources (DiCarlo *et al.*, 1953).

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 63 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 may exhibit B_{12} deficiencies (Goldstein & 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 &

Pateman (1980) described a mutant of *Aspergillius nidulans* for which, with urea as the sole nitrogen source, 0.1 mM Ni^{2+} was required, but 1 mM Ni^{2+} was toxic. 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 a carcinogenic metal (Dosanjh & 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 MccA 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 to form 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-keto acids cannot be redirected into central metabolism and are instead decarboxylated, reduced, and excreted (Hazelwood *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 colleagues. They found that Δ*mcc* strains of *A. nidulans* could not grow on leucine as the sole carbon source and accumulated 3-hydroxyisovaleric acid in the culture supernatants. 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 elevated in media containing 30 mM of leucine, valine, isoleucine, methionine, or phenylalanine, but not in any of the other four amino acids tested (Rodriguez *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) allows 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 Mühlschlegel's group (Klengel *et al.*, 2005; Bahn & Mühlschlegel, 2006), 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 generated 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* (D. Navarathna & D. Roberts, unpublished data).

Acknowledgements

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