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Abstract: Amongst 1.5 million fatal mycoses of humans occurring annually [1], the vast majority involve the human lung as the primary site of pathogenesis, and are derived from organisms which occupy environmental niches. On entry into the respiratory system pathogenic fungi must draw upon metabolic versatility for survival and proliferation as the mammalian lung is a nutritionally limiting environment. The nutritional stresses encountered have exposed vulnerabilities which have long been viewed as potential antifungal targets, since humans lack several of the metabolic pathways which fungi rely upon for pathogenic growth. However the ability of saprophytic fungi to proteolytically liberate amino acids from exogenous protein sources, and the differential availabilities of amino acids in diverse host niches have undermined confidence in amino acid metabolism as a target for selectively toxic antifungal therapies. Recent studies have reopened this debate by revealing a number of anabolic amino acid pathways in pathogenic fungi as being essential for viability per se. This review examines new knowledge on fungal amino acid metabolism in fungal pathogens of the human lung with a view to highlighting important new advances and gaps in understanding.

TITLE

Amino acid biosynthetic routes as drug targets for pulmonary fungal pathogens: What is known and why do we need to know more?

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Abstract

Amongst 1.5 million fatal mycoses of humans occurring annually [1], the vast majority involve the human lung as the primary site of pathogenesis, and are derived from organisms which occupy environmental niches. On entry into the respiratory system pathogenic fungi must draw upon metabolic versatility for survival and proliferation as the mammalian lung is a nutritionally limiting environment. The nutritional stresses encountered have exposed vulnerabilities which have long been viewed as potential antifungal targets, since humans lack several of the metabolic pathways which fungi rely upon for pathogenic growth. However the ability of saprophytic fungi to proteolytically liberate amino acids from exogenous protein sources, and the differential availabilities of amino acids in diverse host niches have undermined confidence in amino acid metabolism as a target for selectively toxic antifungal therapies. Recent studies have reopened this debate by revealing a number of anabolic amino acid pathways in pathogenic fungi as being essential for viability *per se*. This review examines new knowledge on fungal amino acid metabolism in fungal pathogens of the human lung with a view to highlighting important new advances and gaps in understanding.

Role of amino acid metabolism in fungal pathogenicity

Throughout the kingdoms of life, amino acids are important building blocks of proteins and critical sources of macroelements such as nitrogen and sulphur which, if not acquired via intrinsic anabolic metabolism, must be sourced from extrinsic sources. Although fungi can biosynthesise all 20 of the proteinogenic amino acids the uptake of exogenous amino acids is significantly energetically preferable [2]. Accordingly, saprophytic fungal genomes harbour a multitude of protease and peptidase, and amino acid transporter encoding genes to ensure the liberation and uptake of amino acids from exogenous proteinaceous substrates.

Amongst the hierarchy of preferred nitrogen sources the nitrogen-rich amino acids glutamine and arginine are dominant, but other amino acids can also be utilised when starvation threatens. Several wide domain regulatory mechanisms operate to ensure that starvation for nitrogen, carbon or amino acids is met with an appropriate metabolic response. In response to intracellular glutamine levels AreA-type GATA factors interact with other transcription factors specific for the biosynthesis of alternative nitrogen sources eg proline [3,4], while amino acid starvation increases translation of the transcriptional activator CpcA to generate a broad-acting cellular response governing amino acid biosynthesis and uptake [5]. Cellular levels of cysteine and methionine reflect intracellular sulphur

content and modulate sulphur uptake via sulphur metabolite repression involving the positive-acting Cys3/MetR transcription factor and the negative-acting Scon proteins. Recently these regulators have been found to be important for fungal pathogenicity [6-8] (and references therein).

In support of the view that efficient responses to amino acid starvation promote fungal pathogenicity in the mammalian lung Krappmann and colleagues showed that a null mutant of the *A. fumigatus* CpcA transcriptional regulator shows reduced virulence in a leukopenic murine model of pulmonary aspergillosis [5] and Hensel *et al* demonstrated that nitrogen metabolite repression via *A. fumigatus* AreA was required for full pathogenicity in a similar murine model [3]. However, in *Cryptococcus neoformans* the Gat1 transcription factor, regulating nitrogen metabolite repression as well as catabolic enzyme and permease-encoding genes is also a negative regulator of virulence traits such as melanin production and thermotolerance. As a result of these phenotypes a Gat1 null mutant is slightly more virulent than the wild type [4]. These data suggest that the ability to fine tune routes of macronutrient acquisition has an impact upon success of the pathogen in the host niche. However, if fine tuning of amino acid biosynthesis were alone sufficient to support pathogenic growth, fully attenuated (i.e. avirulent) phenotypes would be expected from regulatory mutants, which is not universally the case. A growing body of new data discussed in this review provides strong support for the theory that beyond merely satisfying nutritional requirements, further pleiotropic consequences of ablating amino acid biosynthesis exist which can more critically impact pathogenicity.

Role of amino acid biosynthesising gene products in fungal pathogenicity

Amongst the multiple gene products involved in fungal amino acid biosynthesis, roles for many in pathogenesis have been described. We have classified the encoded enzymatic activities into three phenotypic groups: those which are conditionally essential for pathogenicity (dependent upon host niche); those which are essential for pathogenicity (independent of host niche); and those which are essential for fungal viability *per se*.

Niche-specific requirements for fungal amino acid biosynthesis in pathogenicity

For inhaled fungal pathogens it is sometimes the case that a nutritional requirement essential for survival and proliferation in the pulmonary niche is not replicated when the respiratory niche is bypassed by injecting the pathogen directly into the bloodstream, or via the intrathecal (spinal) route. For example, experimental infections with glycolysis/gluconeogenesis mutants of the inhaled fungal pathogen *Cryptococus neoformans* revealed that entry of 2- and 3-carbon substrates into

gluconeogenesis is required for pathogenicity in an inhalational model of disease, but dispensable for persistence in cerebrospinal fluid [9]. In the same study mutants impaired for glucose utilisation demonstrated decreased persistence in cerebrospinal fluid (CSF) but wild type virulence in an inhalational model of cryptococcosis. From a therapeutic perspective this type of niche-specific phenotype creates significant cause for concern because progression from a pulmonary to a disseminated or cerebral infection would be synonymous with treatment failure. However, despite the obvious importance of deciphering niche-specific pathogenicity phenotypes, it is not commonplace to do so in experimental studies of fungal pathogenicity factors. For example, none of the non-essential amino acid biosynthetic gene products required for *Cryptococcus* pathogenicity (detailed below) have thus far been additionally tested in systemic or CSF persistence models (Table 1). For studies of *Aspergillus fumigatus* several amino acid biosynthetic gene products have been more rigorously addressed, a pivotal study being that of a homocitrate synthase enzyme (HscA), required as the first pathway-specific step (Figure 1) of the lysine biosynthetic α-aminoadipate pathway [10]. Although an HscA null mutant was found to be auxotrophic for lysine biosynthesis, *in vitro*, and unable to undergo spore germination in media containing unhydrolysed proteins, significant growth of the mutant was observed on blood agar and serum-containing growth media. Moreover, if pre-germinated in nutrient-sufficient media, mutant hyphae were able to grow on protein-containing media. These simple *in vitro* tests revealed that HscA null spores, but not hyphae, require free lysine for growth and further experimentation supported a role for hyphally-derived proteases in liberating sufficient lysine for hyphal growth. Finally, analyses of pathogenicity revealed opposing results of avirulence and full virulence, respectively, in inhalational and disseminated models of aspergillosis suggesting differential availability of lysine in these distinct host niches. Similarly in *A. fumigatus*, an AroC null mutant lacking chorismate mutase or a TrpA null mutant lacking anthranilate synthase, which are respectively auxotrophic for both tyrosine and phenylalanine, or for tryptophan, are avirulent in a murine neutropenic model of pulmonary infection but only partially attenuated in a systemic model of disease [11]. Converse niche-specific phenotypes have also been reported, an *A. fumigatus* isoleucine/valine auxotroph lacking the dihydroxyacid dehydratase *ilv3A* encoding gene [12], is avirulent in systemic infection, but only slightly attenuated in pulmonary infection. Notably, further deletion of the paralogous ilv3B gene, previously shown not to be required for prototrophy, led to avirulence also in a pulmonary model of infection [12].

Niche-independent requirements for fungal amino acid biosynthesis

Dietl *et al* showed that an *A. fumigatus* HisB null mutant, lacking a putative imidazoleglycerol phosphate dehydrogenase is auxotrophic for histidine biosynthesis, and unable to grow in blood agar medium or with hydrolysed or non-hydrolysed bovine serum albumin (BSA) as nitrogen source *in vitro*. The mutant was found to be unable to germinate in the lungs of neutropenic mice and was also avirulent in a systemic model of infection [13], suggesting that the amount of available histidine in the lung nor in serum is insufficient to support fungal growth. Interestingly, the *ΔhisB* mutant also demonstrates reduced tolerance of metal excess or iron starvation. This latter finding highlights that amino acid auxotrophy may not always be the sole cause of virulence attenuation as some anabolic routes also impact other metabolic processes which are critical for fungal virulence.

Essential genes in fungal amino acid biosynthesis

An emerging group of anabolic gene products having particular interest as antifungal targets are amino acid biosynthetic genes having apparently essential roles in fungal viability. To date, and to the best of our knowledge, there are published studies on eight relevant fungal genes (Table 1), all of which relate to amino acid biosynthesis in *A. fumigatus* or *C. neoformans*.

Acting upon important roles found for the *A. fumigatus* AroC and TrpA gene products in pulmonary and disseminated infections [11], Sasse *et al* attempted to construct a mutant having a triple auxotrophy for aromatic acid (phenylalanine, tryptophan and tyrosine) biosynthesis. When it proved impossible to generate a viable mutant lacking both of the *aroC* and *trpA* gene products the authors instead focused upon an upstream multifunctional component, AroM (Figure 1). An AroM null mutant also proved unobtainable, thereby inferring essentiality of the shikimate pathway in *A. fumigatus*. Using a conditional promoter driving expression of the AroB gene product (chorismate synthase) a triple auxotroph was obtained, the growth of which could not be rescued by exogenous supply of aromatic amino acids, and which was insufficient to promote full virulence in pulmonary and systemic models of murine infection. The authors hypothesised that this might be due to a deficiency in the collective transport of aromatic amino acids; an alternative hypothesis might be a toxic accumulation of chorismic acid, which inhibits mitochondrial function. Genes *TRP3* and *TRP5* of the *C. neoformans* tryptophan biosynthetic pathway, have also been reported to be essential but their roles in pathogenicity remain untested [14], as do those of all *C. neoformans* amino acid biosynthetic gene products thus far found to be essential *in vitro*. It is important to note that (as for studies of aromatic amino acid biosynthesis in *A. fumigatus* where tetracycline-responsive promoter tools have been developed [15-17]) the ability to harness regulatable expression of the essential

gene products during mammalian infection is a critical step in discerning utility as antifungal drug targets because such an approach allows investigators to formally rule out complementation of auxotrophies via uptake of exogenous amino acids in the host niche. For example, in the case of *C. neoformans TRP3* and *TRP5* genes, *in vitro* supplementation of null mutants with exogenous tryptophan under nitrogen catabolite depressing conditions (proline as nitrogen source) led to partial rescue of growth, presumably via upregulated tryptophan uptake. A similar scenario is relevant to threonine biosynthesis in *C. neoformans* [18] where null mutants lacking any of three gene products (*HOM3*, *THR1*, *THR4*) respectively encoding aspartate kinase, homoserine kinase and threonine synthase) proved recalcitrant to deletion. Using a copper-repressible promoter the *HOM3* and *THR1* genes were further proved to be essential for growth at physiological temperature even in the presence of threonine and methionine, however, growth of conditional mutants could be rescued by threonine dipeptides in the presence of proline as sole nitrogen source. Taking into account that expression of amino acid permeases is upregulated during macrophage infection [19], the actual relevance of threonine biosynthetic genes for *C. neoformans* virulence needs to be confirmed *in vivo*.

Seeking the in-host source of sulphur, an essential macronutrient, during *A. fumigatus* infection Amich *et al* explored the use of the sulphur containing amino acid methionine [20]. Following an unsuccessful attempt to construct a MetH null mutant lacking the *A. fumigatus* methionine synthase, the construction of a conditional expression strain confirmed essentiality of the methionine synthase gene product for *A. fumigatus* viability, and growth of a conditional null mutant could not be rescued by provision of exogenous methionine, even in under nitrogen limiting growth conditions. Growth could, however, be rescued via supplementation with casamino acids and an excess of methionine, but despite this was found to be critical for pathogenicity in a leukopenic model of murine pulmonary infection. In *S. cerevisiae* methionine and SAM levels are thought to represent a critical gauge of amino acid availability that is sensed via methylation modification of PP2A to reciprocally regulate cell growth and autophagy [21]. Fine tuning of methionine biosynthesis might therefore be required for accurate control of methionine and SAM levels and thus for correct functioning of amino acid sensing. During the course of this study, using a double deletion mutant lacking the MecA cystathionine-β-synthase and a CysB cysteine synthase and therefore blocked in both the major and alternative pathways of cysteine biosynthesis, the authors also demonstrated that the S-containing amino acid cysteine is limiting for infectious *A. fumigatus* growth.

Pathways where further characterisation of fungal amino acid biosynthesis is needed

Mammals depend upon dietary sources for acquisition of nine proteinogenic amino acids (phenylalanine, valine, threonine, tryptophan, isoleucine, methionine, leucine, lysine and histidine) making the corresponding microbial biosynthetic pathways attractive targets for selectively toxic therapies. As a result of our literature survey we have identified a total of fourteen relevant fungal gene products, acting in any one of these biosynthetic pathways and having been demonstrated as important for pulmonary fungal infection, of which four derive from *C. neoformans* and ten from *A. fumigatus*. In addition, eight gene products have been demonstrated as being essential for viability, five from *C. neoformans* and three from *A. fumigatus*. In common amongst the essential phenotypes of these two species are gene products acting to biosynthesise the aromatic amino acids. Thus far essential requirements for methionine and threonine biosynthesis appear restricted, respectively, to *A. fumigatus* and *C. neoformans*.

Despite the heavy burden of fatal mucormycoses, pneumocystoses, penecillioses, coccidiomycoses, blastomycoses and paracoccidiomycoses [1] we have failed to find any relevant studies in the causative organisms. Several dimorphic species have long been known to be organic sulphur auxotrophs in their pathogenic yeast forms, namely *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Penicillium brasiliensis* [22-25]. This of course points to methionine and cysteine as sources of sulphur in the lung environment, a hypothesis supported by the observed upregulation of the *P. brasiliensis* high affinity methionine permease under infective conditions [26]; however, and surprisingly, to our knowledge the actual importance of cysteine and methionine assimilation for virulence of those fungi has not been investigated. Clearly, a great deal of new research into amino acid biosynthesis in all of these organisms is long overdue if the broad spectrum utility of targeting such pathways is to be clarified.

For *C. neoformans*, all five essential genes need validation in animal models and four non-essential gene products have been demonstrated as critical in the pulmonary host niche but remain untested in systemic or cerebrospinal infections (Table 1). In particular, methionine auxotrophs in *Cryptococcus neoformans* lacking either of the Met6 (methionine synthase) or Met2 (homoserine transacetylase) gene products, or mutants lacking the *C. neoformans* acetolactate synthase encoding gene *ILV2* (resulting in isoleucine/valine auxotrophy) all of which are avirulent in inhalational models of disease [27-29]. Cryptococcal leucine biosynthesis from 2-ketoisovalerate, a biosynthetic pathway absent in humans and requiring the isopropylmalate dehydrogenase Leu1 is required for full virulence in an intranasal murine model of cryptococcosis but remains untested in a cerebrospinal model [30]. Interestingly, branched chain amino acids are precursors for fatty acid synthesis [31, 32],

which is an essential and quite unexplored field in fungi. For *A. fumigatus*, the pathogenicity of *aroM*, *metH* and *cysB;mecA* null mutants awaits further validation in systemic infection, and an *argEF* mutant, of the arginine biosynthesis pathway, which showed decreased virulence in an insect model of infection [33] remains to be tested in animals. The correspondent gene product forms ornithine, which is the major precursor of siderophores [34], and of essential polyamines [35, 36], although in the presence of arginine the effect on pathogenicity was ascribed to the reduction in virulence-essential [37] siderophores[33].

Perspectives for drug development

A wide range of chemicals inhibit enzymatic activities involved in amino acid biosynthesis, as recently reviewed by Jastrzębowska and Gabriel [38]. Unfortunately, the compounds either have not thus far been tested, or have showed limited effect, in animal models. Nevertheless, most of these compounds have demonstrated very little, if any, mammalian toxicity and are able to overcome fungal multidrug resistance, which are two desirable features for novel antifungal drugs. Therefore, we believe that identification of drug targets in amino acid metabolic pathways and the search for specific inhibitors holds promise for revolutionising antifungal treatment.

As well as being critical for *A. fumigatus* and *C. neoformans* pathogenicity, aromatic acid biosynthesis is required for *Mycobacterium tuberculosis* pathogenicity and the *M. tuberculosis TRP3* gene product (3-indol-glycerol phosphate synthase) is druggable [39], as are tryptophan biosynthetic enzymes of various other microorganisms [40-43], raising the intriguing prospect of developing broad spectrum agents which have antimicrobial relevance beyond targets of the fungal kingdom.

Considerable controversy surrounds the suitability of amino acid metabolism as a target for antifungal treatment. The observations of Schöbel and colleagues revealed that an *A. fumigatus* lysine auxotroph is able to germinate and grow on partially digested proteinaceous substrates [10], which is also the case for an *A. fumigatus* cysteine auxotroph [20]; moreover the mutant is fully virulent in a disseminated disease model. These observations highlight two critical caveats of targeting amino acid biosynthesis. First, some auxotrophs can sufficiently supplement their growth programmes by uptake of exogenous amino acids and second, that niche-specific nutritional requirements can differ dramatically. Thus, in the case of specific amino acids, treatments aimed at established invasive disease might not be effective were the pathogen able to access amino acids *in vivo* by the secretion of proteases that release amino acids from host proteins. Although this assumption seems plausible, it has not been formally tested; indeed the requirement for protease secretion for fungal virulence, despite recently published insights [44,45], currently remains unclear.

Interestingly, metabolically active, protease secreting *Cryptococcus neoformans* yeast cells having methionine or isoleucine/valine auxotrophies are all avirulent [45], although some studies suggest that uptake of amino acids might not be very efficient in this pathogen [14,18], which would explain the frequent avirulence of auxotrophic strains. In contrast, the obligate pathogen *Pneumocystis jirovecii* which needs to acquire the amino acids from host lung tissue seems to secrete proteases [46]. To better address questions surrounding *in vivo* acquisition of amino acids essential for fungal viability novel genetic tools which allow temporal manipulation of gene expression during infection are required. A good example is the Tet-OFF system, recently established in *A. fumigatus* [17]. Although it still waits validation in animal models, the advantage of this system is that it permits shut-down of gene expression at specific time-points during infection; thereby better approximating the delivery of an antifungal drug to an established infection.

On the basis of current knowledge, the superior strategy appears to be that of targeting processes which are essential for fungal viability and/or that have a role beyond amino acid metabolism. Impairment of such enzymatic activities would not only impose the acquisition of the given amino acid, but would abrogate growth in the lung environment. However, it would be important to clarify the precise mechanistic basis of the virulence defect before further advancing in the development of novel therapies. In this regard, we believe that current understanding of fungal amino acid metabolism in the mammalian lung environment is limited and very fragmented. First of all, most of the information comes from the two major pathogens *A. fumigatus* and *C. neoformans*. Besides, the pathways investigated differ considerably between pathogens, and even the genes studied within one pathway are often not the same. To our knowledge, the relevance of the biosynthesis of several amino acids (Ser, Asn, Asp Gln, Gly, Glu, Pro and Ala) has not been investigated at all in pathogenic fungi, which also prevents a complete understanding of the *in vivo* situation.

Highlights

Amino acid biosynthetic pathways are attractive drug targets since many of these pathways are conserved in microbes but absent from humans.

Amongst the 20 proteinogenic amino acids, the biosynthesis of eight have been studied in pathogenic fungi, and eight relevant gene products have been identified as essential for fungal viability

When potential therapeutic targets come under experimental scrutiny, testing of niche-specificity of pathogenic phenotypes should be commonplace. Currently it is not.

Much more work required in amino acid biosynthesis in pathogenic fungi, In particular in the dimorphic species.

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Figure 1. Schematic overview of fungal amino acid biosynthetic pathways.

An abbreviated version of the biosynthetic routes, in fungi, of all 20 proteinogenic amino acids. All genes investigated to date (as discussed in the text) are shown. Genes are discriminated by fungal species, the host niches in which they are known to be required for pathogenicity, and by essentiality for fungal viability, as described in the legend. Amino acids framed in purple are essential nutrients in humans which must be sourced from the diet as the capacity to biosynthesise them is absent, thereby highlighting the relevance of the corresponding fungal biosynthetic routes as prospects for for drug development.
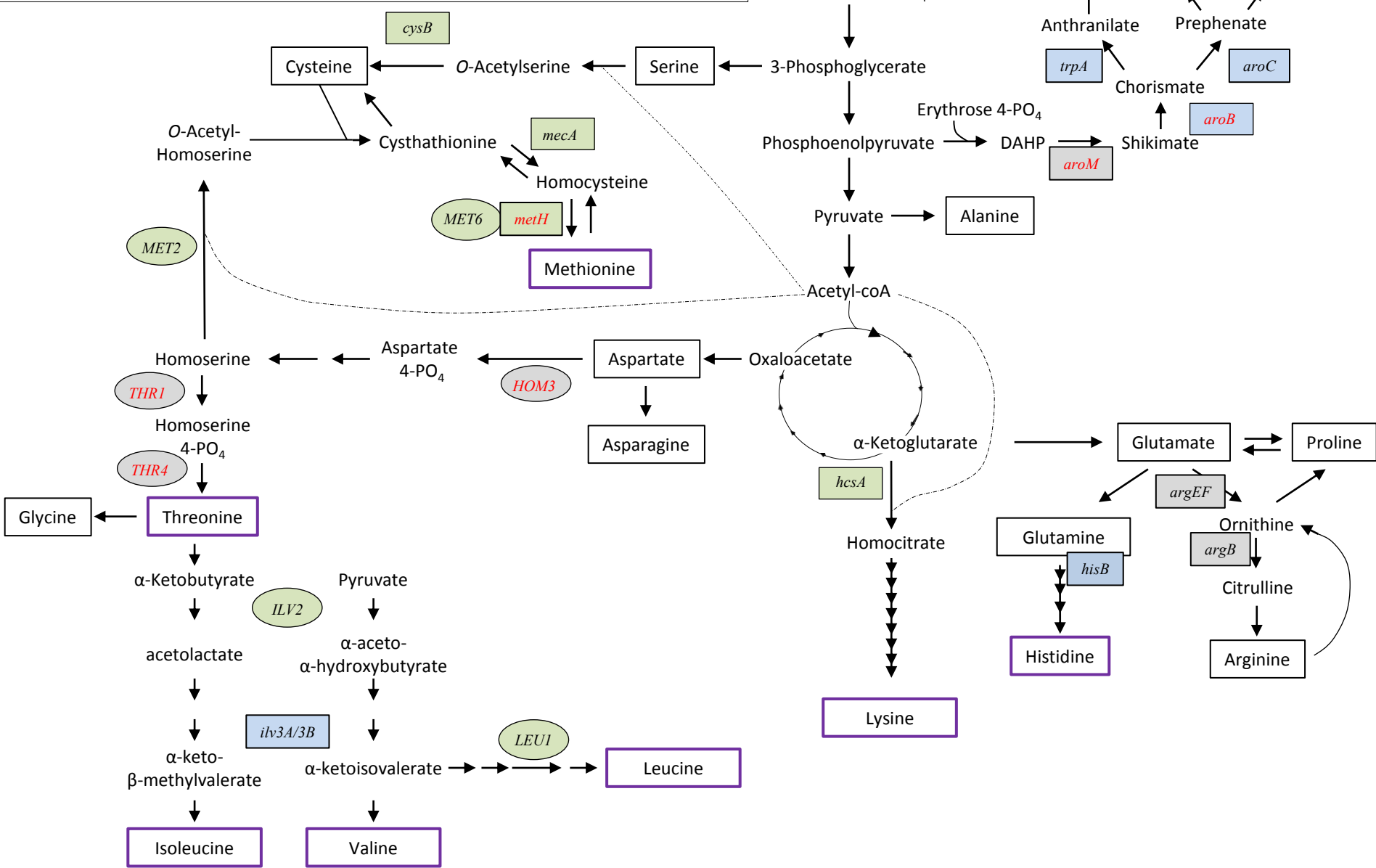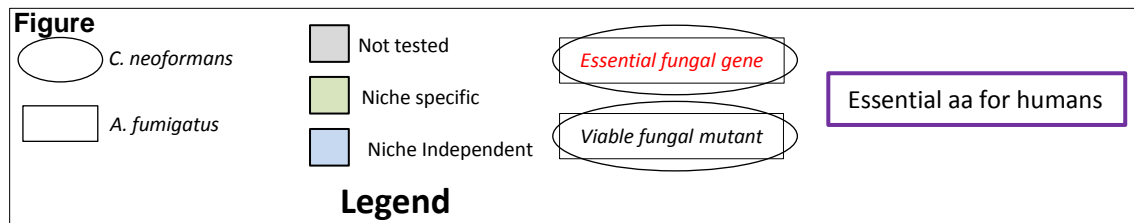
Highlights

Amino acid biosynthetic pathways are attractive drug targets since many of these pathways are conserved in microbes but absent from humans.

Amongst the 20 proteinogenic amino acids, the biosynthesis of eight have been studied in pathogenic fungi, and eight relevant gene products have been identified as essential for fungal viability

When potential therapeutic targets come under experimental scrutiny, testing of niche-specificity of pathogenic phenotypes should be commonplace. Currently it is not.

Much more work required in amino acid biosynthesis in pathogenic fungi, In particular in the dimorphic species.

Figure

Legend:
- *C. neoformans* (ellipse)
- *A. fumigatus* (rectangle)
- Not tested (grey)
- Niche specific (green)
- Niche Independent (blue)
- *Essential fungal gene* (ellipse, red italic)
- *Viable fungal mutant* (ellipse)
- Essential aa for humans (purple box)

Detailed Response to Reviewers

➤ Reviewer #1: The manuscript presents a review of the current literature relating to amino acid biosynthesis as a target for anti-infective therapy in pulmonary fungal pathogens. The authors present an interesting narrative, highlighting that amino acid biosynthesis pathways have been somewhat overlooked as a source of targets for therapy. They argue this is primarily due to an inference from early research that suggested Aspergillus strains lacking the capability to synthesise lysine are still virulent due to their ability to liberate amino acids from lung tissue via the action of secreted proteases. They draw comparisons from recent publications to suggest that the biosynthesis of some AA may be more critical for virulence than others particularly in certain infection niches. The authors also cite recent literature that shows an interdependency between amino acid biosynthesis and other critical virulence factors such as micronutrient acquisition and melanin biosynthesis.

They argue that there is significant need for further exploration of AA biosynthesis in pathogenicity. The manuscript is well written and represents the current literature accurately. This review is timely given our increased understanding of the importance of fungal infections, rising levels of drug resistance and a dearth of antifungal agents.

Response: We thank the reviewer for the positive revision of the review and its formative objective.

Required correction

➤ There seems to be a discrepancy between the table and the figure with respect to the pathways that are defined as essential or viable. The authors should check this.

Response: We thank the reviewer to point out this source of confusion. We have now clarified the figure legend to highlight that the framed amino acids are essential for humans.

I would suggest some other changes that the authors should consider:

➤ While the purpose of the manuscript is to review the literature relating to pulmonary fungal pathogens, it may be useful to draw comparisons with available data from Candida species where lysine and histidine biosynthesis seem dispensable for virulence. This would also better reflect the current broad title of the manuscript.

Response: We have focused on pulmonary fungal pathogens and therefore, *Candida* species are out of the scope of this review. *Candida* species are primarily commensals of the human gut and thus

their access to dietary amino acids as well as their genetic requirements for biosynthesis radically differ from lung pathogens. We thank the review for his suggestion, but we consider that due to that reason, comparisons cannot be directly drawn. We agree that the title was too broad, and have changed it to emphasise the focus on pulmonary pathogens.

➢ The authors state that ornithine, the product generated from the action of ArgEF, is a precursor for the generation of arginine and siderophores. The authors should also stress that ornithine is also a precursor for polyamines, which are critical for fungal viability (Rajam et al 1985; PNAS 82 6874-amongst others).

Response: We agree with the reviewer that polyamines should be mentioned here as essential derivatives of ornithine, and have included this information. However, we also state that the reduction in virulence that Beckmann et al observed was ascribed to the decrease in siderophore, rather than polyamines.

➢ There is a body of literature from bacteria and mammalian systems that highlight crosstalk between branched chain amino acid biosynthesis and lipogenesis. The authors may want to draw attention to this relatively unexplored area of fungal metabolism (Beck, FEMS microbiology letters 2005 Feb 1;243(1):37-44, Crown et al, Plos one http://dx.doi.org/10.1371/journal.pone.0145850).

Response: We thank the reviewer for this suggestion. We have included a sentence to draw attention on this important and unexplored field.