Medical Mycology April 2011, **49**(Suppl. 1), S107–S113

informa
healthcare

Pathogenicity of *Aspergillus fumigatus* mutants assessed in *Galleria mellonella* matches that in mice

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Aspergillus fumigatus is a clinically important fungus with the ability to cause invasive aspergillosis with high mortality rates in immunocompromised patients and chronic pulmonary aspergillosis in immunocompetent individuals. Virulence of mutants has traditionally been assessed using mammalian hosts such as mice and rats and more recently the fruit fly, *Drosophila melanogaster*, demonstrated the potential to act as an *in vivo* host suitable for screening *Aspergillus* mutants. In this study using a larger thermotolerant invertebrate, *Galleria mellonella*, the virulence of individual gene deletants of *Aspergillus fumigatus* (*cpcA*, *sid*A, *sid*C, *sid*D, *sid*F and *paba,*) were compared to the parental and gene-replacement strains, if available. A range of infectious challenges consisting of from 3×10^3–3×10^6 spores/larva was followed by observation of larval survival with mean survival time used as a surrogate of microbial pathogenicity. Mutants *cpcA*, *sid*A, *sid*F and *paba* were avirulent and *sid*C and *sid*D showed attenuated virulence. Virulence assessment in *G. mellonella* correlated closely with the historic data generated using mice and *Drosophila*. Pre-screening *Aspergillus* mutants using *G. mellonella* could significantly reduce the number of mammals required to assess changes in virulence.

Keywords *Galleria mellonella*, wax moth larvae, siderophore, invasive aspergillosis, *Drosophila melanogaster*

Introduction

Aspergillus fumigatus is an opportunistic, saprophytic pathogen which is the leading worldwide cause of invasive aspergillosis (IA) in immunocompromised patients [1], as well as causing chronic disease and allergy in immunocompetent patients. Assessment of the pathogenicity of mutant strains of *A. fumigatus* is critical in defining virulence factors associated with the fungus. In addition, virulence factors have been proposed as potential targets for novel therapeutics. Virulence of microbial pathogens has traditionally been determined using lethal infections in mammalian hosts such as mice or rats. Mammalian models are regarded as the gold standard [2] for virulence assessment of clinically important pathogens due to their similarity to humans in their immune function and disease progression. However, mammalian models of infectious disease are expensive, ethically complicated and require specialized facilities. Invertebrate hosts have been employed for *in vivo* screening of virulence, for example *Drosophila melanogaster* and *Caenorhabditis elegans*, both of which are genetically tractable insects which have been used primarily in biological research (genetics and developmental biology) due to their rapid life cycle. In virulence studies, *D. melanogaster* has been utilized to screen the *Burkholderia cepacia* complex (Bcc) [3], while *C. elegans* was employed in studies of *Pseudomonas aeruginosa* [4]. However, both are small (<2.5mm), pose technical difficulties for the accurate delivery of infectious challenges, and do not tolerate the physiological temperatures of human hosts.

G. mellonella larvae (Greater wax moth) are 20 mm in length and weigh 0.2 g making them large enough for accurate parenteral dosing. Additional major benefits are their ability to thrive at 37°C, ease of maintenance and manipulation, and few ethical constraints. *G. mellonella* have been used to assess the virulence of *Pseudomonas aeruginosa* [5], *Staphylococcus aureus* and the pathogenicity of *Candida* [6,7] with outcomes correlating with murine models. There have been studies which employed *G. mellonella* to screen

Received 19 April 2010; Received in final revised form 1 September 2010; Accepted 11 September 2010

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DOI: 10.3109/13693786.2010.523852

virulence factors of *A. fumigatus*, for example, the assessment of gliotoxin [8], and colour mutants [9] by LD_{90} following parenteral administration.

A wide range of mutant *A. fumigatus* strains have been investigated for virulence in rodent models of disseminated disease to unravel complex metabolic pathways and identify potential novel drug targets. The aim of this paper was to assess the virulence in *G. mellonella* of a small selection of well characterized *A. fumigatus* mutants that have previously been screened for virulence in mice and *D. melanogaster*. The mutants were selected as they demonstrated a spectrum of phenotypes in mice that would be desirable to replicate in *G. mellonella*. The effect on virulence was assessed using mean survival time in *G. mellonella* following parenteral administration of the mutated, parental or reconstituted strains. Survival of the larvae was compared to that seen in similar studies in mice and *D. melanogaster*.

Methods

Isolates

Twelve *A. fumigatus* isolates were investigated which included three wild type strains (AF237, D141 and ATCC 46645 [NCPF 2109]), six strains with gene deletions (Δ*sid*A, Δ*sid*C, Δ*sid*D, Δ*sid*F, Δ*paba and* Δ*cpcA)* and paired reconstituted strains (Table 1) [10,11,14–16]. Strains were recovered from long term storage at −80°C by inoculating them onto Sabouraud dextrose agar (Oxoid, Basingstoke, UK) and incubating at 37°C for 7–10 days. Spores were harvested by flooding the surface with phosphate buffer solution (PBS; Invitrogen, Paisley, UK) plus 0.05% Tween 80 (Sigma, Poole UK), with concentrations estimated through the use of a haemocytometer and confirmed by quantitative culture. Strains were independently randomized and blinded ensuring no bias during survival analysis.

Table 1 Parental, mutant and reconstituted strains used to study and compare the virulence in *Galleria mellonella* of specific gene deletants.

Strain	Genotype and background	Reference
*cpc*A	D141	[14]
D141	WT	[14]
*sid*A	ATCC46645	[15]
*sid*C	ATCC46645	[16]
*sid*F	ATCC46645	[16]
ATCC46645(NCPF 2109)	WT	[15,16]
*cpc*A recon	D141	[14]
paba auxotroph	AF237	[10,11]
*sid*F recon	ATCC46645	[16]
*sid*C recon	ATCC46645	[16]
AF237	WT	[10,11]
*sid*D	ATCC46645	[16]

WT, Wild type parental strain; *sid*, Siderphore; AF, *Aspergillus fumigatus; cpc*, cross pathway control.

Infection studies

Following confirmation of spore density (spore suspensions stored at 4°C for a maximum of 48 h before use), *G. mellonella* individuals were infected by parenteral injection of 10 µl of the suspensions into the last pro-leg of larvae using a Hamilton syringe (Hamilton Company, Switzerland) [17]. Preliminary experiments were performed with the parental strains to define an appropriate range of inocula concentrations that would cause 90–100% mortality at 96 h post infection (data not shown). Based on these data, the infectious challenges required were ~3×10^6, 3×10^5, 3×10^4 and 3×10^3 spores/larva for all parental strains. Groups of 10 larvae were infected with 10 µl of each inoculum suspension, with post infection counts performed to confirm the spore counts. Additional control groups of a total of 30 larvae (10 unmanipulated, 10 pierced and 10 injected with 10 µl of PBS) were also included. Larvae were incubated at 37°C for up to 7 days and survival recorded daily. All survival experiments were performed on three separate occasions. End points were characterized by lack of movement, dehydration and/or melanization of the cuticle. Virulence was defined as <15% survival of larvae 6 days post infection at the highest inocula concentrations tested. Attenuated virulence was defined as a significant difference in survival of larvae compared to the wild type strain, along with an increase in mean survival time compared to the wild type strain at the highest inocula levels studied. Avirulence was defined as a mean survival time of 6 days at the highest inocula concentrations.

Statistics

Mann-Whitney U tests were performed to compare the survival post infection between parental, mutant and reconstituted strains. Kaplan-Meier survival analysis was used to estimate the mean survival time post infection and 95% confidence intervals (Table 2). Statistical analyses were completed using Stats Direct (Altrincham Manchester UK).

Results

All wild type *A. fumigatus* strains were highly virulent causing 90–100% mortality following parenteral administration of 10^5–10^6 spores/larva. Disease progression was marked by increased melanin pigmentation initially at the site of administration, which spread throughout the entire larva 12–36 h before death. At all times the cuticle of the larva remained intact and there was no hyphal penetration through the insect surface.

Of the six *A. fumigatus* mutant strains previously tested in mice, three were characterized as demonstrating attenuated virulence (*cpc*A, *sid*F, and *sid*C) and three were

Table 2 Mean survival times (days) of *Galleria mellonella* larvae infected with six mutant strains of *Aspergillus fumigatus* ($n = 3$) at inocula of either 2–4 $\times$ 10^6 or 2–4 $\times$ 10^5 CFU/larva compared with their parental strain and reconstituted strain if available.

		Mean survival time (days)					
		Mutant	95% CI	Wild type	95% CI	Reconstituted	95% CI
10^6 CFU/larva	*cpc*A	6	–	2	1.0 – 3.0	2	1.1 – 2.9
	*sid*A	6	–	0.8	0.4 – 1.2	NA	
	*sid*F	6	–	0.8	0.4 – 1.2	3.1	1.8 – 4.4
	*sid*D	4.4	3.3 – 5.5	0.8	0.4 – 1.2	NA	
	*sid*C	3.3	2.3 – 4.3	0.8	0.4 – 1.2	1.8	1.3 – 2.3
	paba	6	–	2.1	1.1 – 3.1	NA	
10^5 CFU/larva	*cpc*A	6	–	4.8	3.7 – 5.9	4.3	3.1 – 5.5
	*sid*A	6	–	3	1.8 – 4.2	NA	
	*sid*F	6	–	3	1.8 – 4.2	4.7	3.6 – 5.8
	*sid*D	6	–	3	1.8 – 4.2	NA	
	*sid*C	5.2	4.4 – 6.0	3	1.2 – 4.0	4.2	3.2 – 5.2
	paba	6	–	4.5	3.3 – 5.7	NA	

NA = not available.

classified as avirulent (*sid*A, *paba* and *sid*D). The virulence of the same six *A. fumigatus* mutants assessed in *G. mellonella* were characterized as attenuated for two strains (*sid*D, *sid*C) which almost matches the results observed in mice. Four strains were classified as avirulent (*cpc*A, *sid*A, *sid*F, *paba*) which in two cases is consistent with the outcomes seen in mice (*sid*A, *paba*). However, two other, *cpc*A and *sid*F, were avirulent in *G. mellonella* whereas they were described as attenuated in mice.

At the highest concentration of inocula suspensions (10^6CFU/larva), all six mutants tested were significantly less virulent than the wild type ($P = 0.0047$–$P = 0.049$; Fig. 1). When administered at lower concentrations (10^5 CFU/larva), the survival of mice inoculated with 3/6 mutant strains remained significantly different to that noted with the wild type ($P = 0.021$; Fig. 2). In contrast, the use of 3/6 mutants caused no significant difference in survival as compared to the wild type, i.e., *cpc*A $P = 0.0699$, *sid*C $P = 0.1218$, *paba* $P = 0.0699$. The reconstituted strains demonstrated similar virulence to the parental strains, where these strains were available. (*cpc*A $P = 0.9184$ and $P > 0.9999$; *sid*F $P = 0.0594$ and $P = 0.2045$; *sid*C $P = 0.6125$ and $P = 0.3823$ for inocula of 10^6 and 10^5CFU/larva, respectively).

Additional survival assessments showed only that the mutant strain *cpc*A remained significantly different from the wild type (10^4CFU/larva $P = 0.0256$) when experiments were performed using lower inocula and a range of $P = 0.0769$–$P = 0.6922$ was generated for all other strains tested (data not shown).

Virulence of mutants in Drosophila

Table 3 summarizes the virulence of the six mutant *A. fumigatus* strains assessed in survival models in mice, *G. mellonella* and toll-deficient *D. melanogaster* [18]. Outcomes assessed in *Drosophila* relative to the virulence of 2/7 mutants did not correlate with those observed in mice, with *sid*F and *sid*C mutants demonstrating virulence in *Drosophila* but attenuation in mice. In contrast the survival outcomes in *G. mellonella* and mice demonstrated excellent correlation with minor differences in outcomes (avirulence in *G. mellonella* but attenuation in mice).

Discussion

Siderophore biosynthesis

Schrettl *et al*. [15] demonstrated that *A. fumigatus* has systems to survive in environments with reduced iron and mobilization of iron by siderophores. At least six different hydroxamate-type siderophores of >10KDa are produced by *A. fumigatus*, the two most important being triacetylfusarinine (TAFC) and ferricrocin (FC) (Fig. 3). These are rapidly synthesized in the presence of serum before proteases are released and are required for initiation of growth. Schrettl *et al*. [16] demonstrated that L-ornithine-N^5-oxygenase (*sid*A), which catalyses the first step of the biosynthesis of hydroxamate-type siderophores is an essential virulence factor [15].

The *sid*A mutant strain was avirulent in *G. mellonella* resulting in 100% survival even at the highest inocula tested. In murine models *sid*A mutants caused modest weight loss in the first 24 h, followed by weight gain with no mortality confirming that *sid*A is vital for *A. fumigatus* virulence in mice [15]. These findings were replicated in *G. mellonella* which survived regardless of infectious dose. *Sid*A is the first gene in the biosynthetic pathway of siderophores, converting L-ornithine to N^5-hydroxy-L-ornithine. In the absence of *sid*A, the conversion will not take place and the

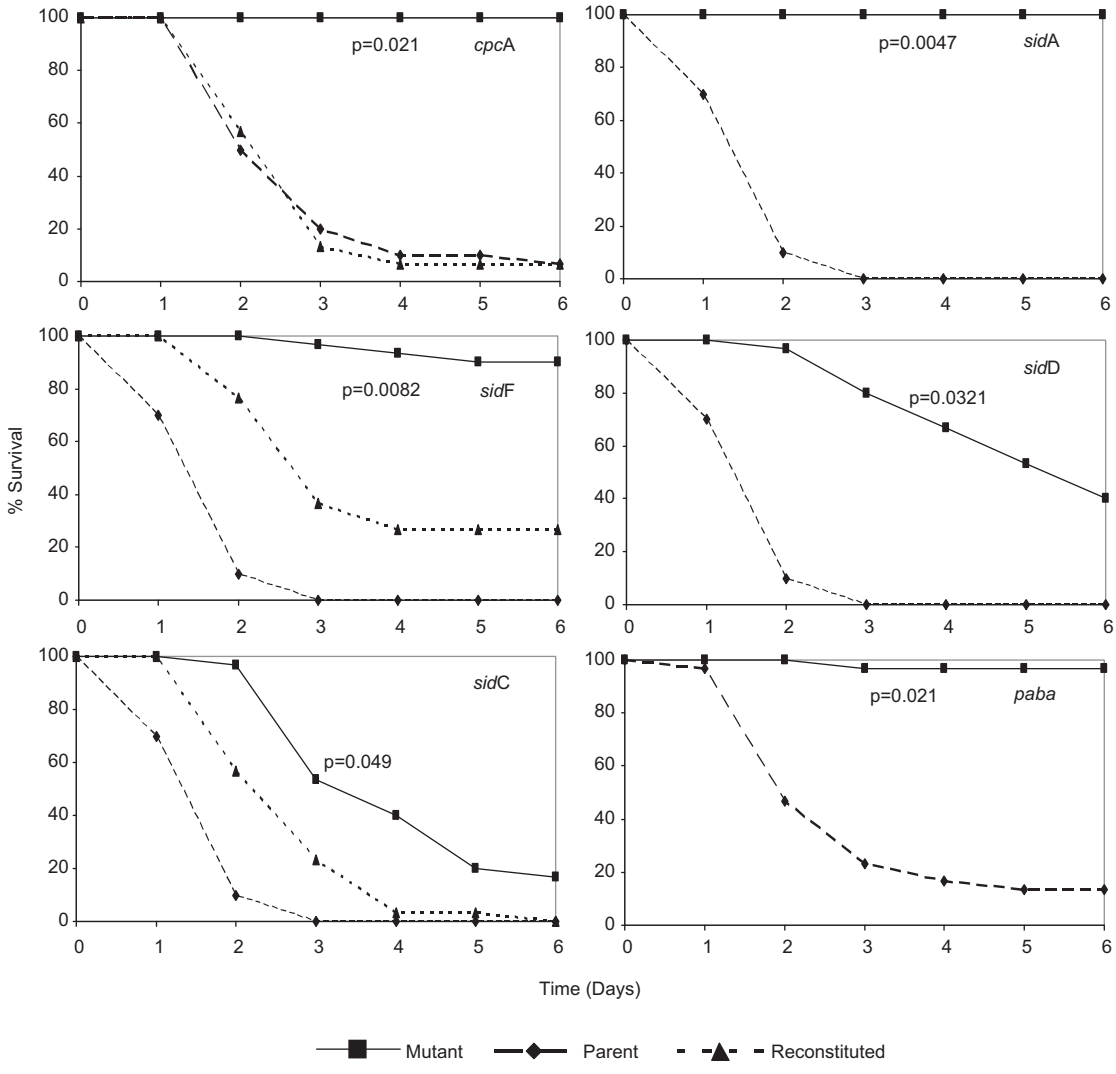

Fig. 1 Mean survival of *Galleria mellonella* larvae infected with 2×10^6–4×10^6 CFU/larva of parental, mutant and reconstituted strains of *Aspergillus fumigatus* (mean of three models). ATCC 46645 was the parent strain for *sid*A, *sid*F, *sid*D and *sid*C. D141 was the parental strain for *cpc*A. AF 237 was the parental strain for the *paba* mutant. The *P* values represent the Mann-Whitney U test statistical difference between larval survival when infected with either mutant or wild type strains.

siderophore production would be halted at an early stage. *sid*A is an essential virulence factor in conditions of low free iron.

The *sid*F gene is the second gene in the siderophore biosynthetic pathway and is essential in conditions of low free iron. Similar to the *sidA* mutant, infection with the *sid*F mutant resulted in almost 100% survival of *G. mellonella*. These data match outcomes in mice where attenuated virulence was observed (36% mortality in mutant infected mice compared to 100% mortality in wild type and complemented strains ($P = 0.0006$)) [16].

Infection with the *Sid*C mutant strain resulted in lower mortality of *G. mellonella* compared to wild type and reconstituted strains indicating attenuated virulence in the invertebrate model. *Sid*C is the third gene in the biosynthetic pathway and converts N^5-acetyl-N^5-hydroxy-L-ornithine into ferricrocin (FC). There is redundancy in this pathway as *Aspergillus* can utilize *sid*D in an alternative pathway suggesting *sid*C is not essential for virulence in low iron conditions. Additionally alternative routes to synthesize siderophores are available as production of TAFC may be sufficient to acquire iron from the environment. We propose that availability of an alternative pathway leads to attenuated virulence (for example the *sidC* mutant) whereas avirulence is the outcome if there is no alternative pathway (for example *sidA* and *sidF* are upstream of an alternative pathway). The outcomes in *G. mellonella* parallel those in mice where *sid*C mutant strains showed attenuated

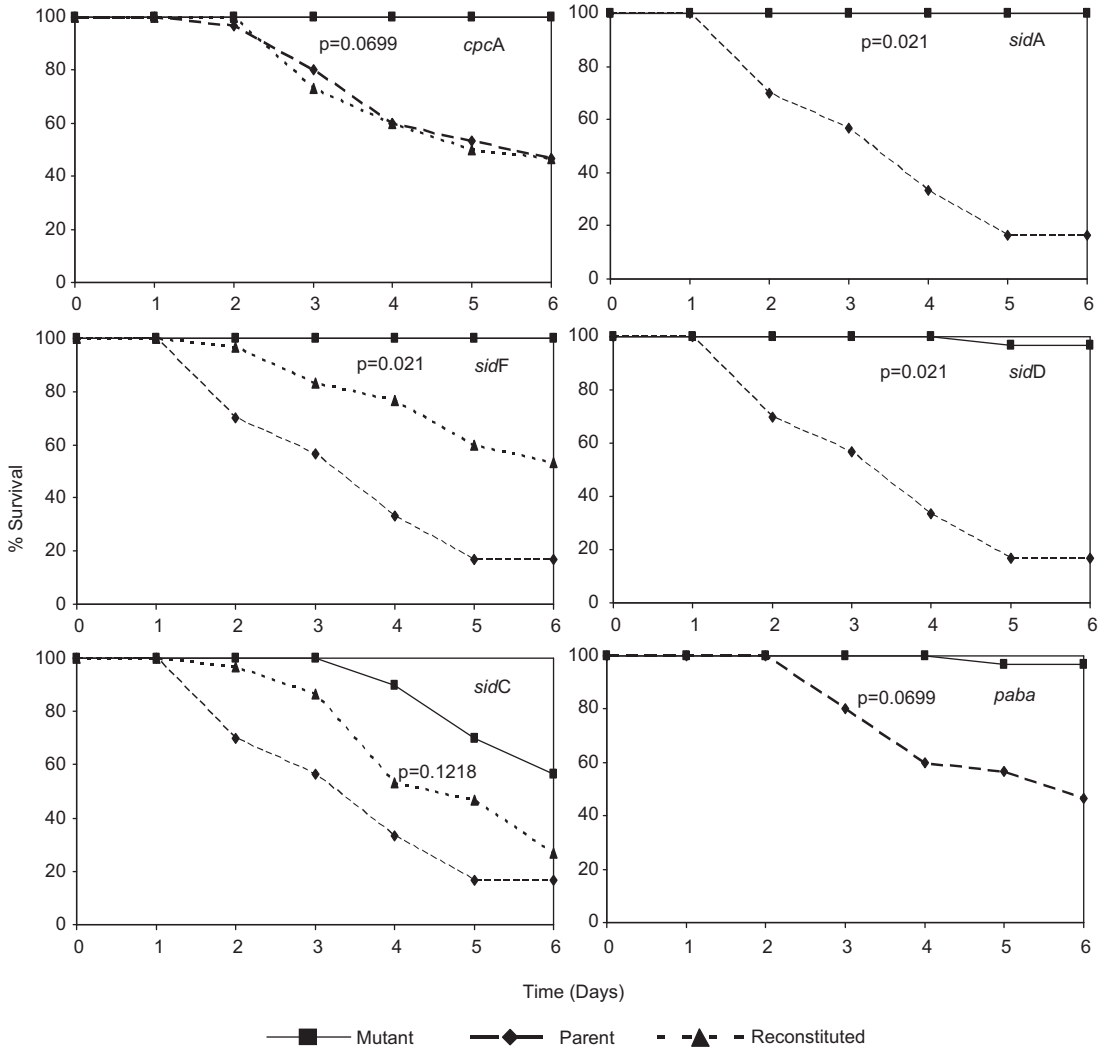

Fig. 2 Mean survival of *Galleria mellonella* larvae infected with 2×10^5–4×10^5 CFU/larva of parental, mutant and reconstituted strains of *Aspergillus fumigatus* (mean of three models). ATCC 46645 was the parent strain for *sid*A, *sid*F, *sid*D and *sid*C. D141 was the parental strain for *cpc*A. AF 237 was the parental strain for the *paba* mutant. The *P* values represent the Mann-Whitney U test statistical difference between larval survival when infected with either mutant or wild type strains.

virulence with significantly higher survival (41% compared to 0% ($P = 0.0017$)) [16].

The *sid*D mutant strain showed attenuated virulence throughout all tested inocula levels. This finding is unsurprising as *sid*D, like *sid*C, is a non-ribosomal peptide synthetase, which converts N^5-*cis*-anhydro-mevalonyl-N^5-hydroxy-L-ornithine to fusarinine C. The alternative pathway facilitated by *sid*C could be used to complete siderophore biosynthesis, and therefore, to aid growth of *A. fumigatus* in low iron environments. In Schrettl's mouse model, the *sidD* mutant was classified as severely, if not completely, attenuated in virulence, with only 1/13 mice possibly succumbing to the infectious challenge ($P = 0.0001$) [16]. It may be more appropriate to say the *sid*D mutant was avirulent in their mouse model. The *G. mellonella* outcomes correlate well with those determined in mice, although the *sid*D mutant caused higher mortality in the *G. mellonella* system.

Data generated in *G. mellonella* support the hypothesis that mutation or deletion of genes encoding early steps in the siderophore biosynthetic pathway has a greater effect on virulence as there are no alternative pathways available. Mutations at the beginning of the pathway resulted in high rates of survival in larvae and mice whereas mutations at later stages in the pathway resulted in attenuated strains.

Cross pathway control

The cross pathway control (CPC) mechanism of *A. fumigatus* involves a transcriptional activator, *cpc*A, the translation

Table 3 Comparison of virulence measured by host survival of six mutant strains compared to wildtype in murine models of disseminated infection [6,11,13,14], *Galleria mellonella* larvae and *Drosophila* [18]. Attenuated virulence defined as a significant difference in survival of larvae compared to the wild type strain along with an increase in mean survival time compared to the wild type strain at the highest inocula tested. Avirulence defined as a mean survival time of 6 days at the highest inocula tested.

Mutation	Mouse	*Galleria mellonella*	*Drosophila*
*cpc*A	Attenuated	Avirulent	Hypovirulent
*sid*A	Avirulent	Avirulent	Avirulent
*sid*F	Attenuated	Avirulent	Not attenuated
*sid*D	Avirulent	Attenuated	Attenuated
*sid*C	Attenuated	Attenuated	Not attenuated
paba	Avirulent	Avirulent	Avirulent

of which is increased during amino acid starvation to produce a global cellular response. Increases in *cpc*A are initiated by *cpc*C protein kinases which phosphorylates the α subunit of a trimeric complex, eIF_2. It has previously been demonstrated that the *cpc*C gene is not necessary for *A. fumigatus* pathogenicity [14] and that the environment encountered by *A. fumigatus* during pulmonary infection does not trigger a CPC response. However, under amino acid starvation, *cpc*A is required for the *CPC* response. The *cpc*A deleted mutants had attenuated virulence in murine models, though no obvious growth phenotype was noted *in vitro* [13]. The findings in mice were mirrored in our studies in *G. mellonella* in which the *cpc*A mutant failed to cause lethal infections.

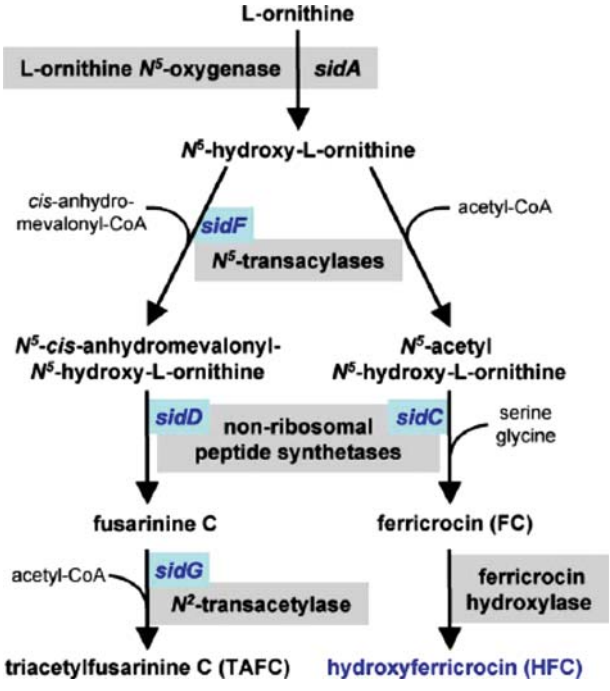

Fig. 3 Postulated Siderophore Biosynthetic Pathway [16].

Folate biosynthesis

The *paba*A gene encodes para-aminobenzoic acid (PABA) synthetase as part of the folate biosynthesis pathway. PABA synthetase is essential for *A. fumigatus* spores to both initiate new infections and maintain existing infections in murine models of invasive pulmonary aspergillosis (IPA) [10]. *paba*A mutants are unable to synthesize folate or grow in the mammalian lung as there is no exogenous source in this environment. Folate is an essential co-factor in DNA synthesis which explains the importance as a virulence factor. It has been suggested that inhibition of the *paba*A product may provide an effective therapy for invasive *A. fumigatus* infections [10].

The *paba*A mutant strain was almost avirulent in *G. mellonella* larva regardless of the inocula concentrations used which correlates with outcomes in murine models of disseminated and pulmonary disease [10]. This data confirms that the *paba*A gene is essential for growth *in vivo* and critical for virulence of *A. fumigatus*.

Overall, two mutants were avirulent in mice, *G. mellonella*, and *Drosophila*. Four mutants were attenuated in mice, but two were attenuated and two avirulent in *G. mellonella*, indicating minor differences in classification. In contrast, two strains were attenuated and two remained virulent (*sid*F and *sid*C) in *Drosophila* indicating significant divergence in classification. It is unclear why the virulence assessed in *Drosophila* did not correlate more closely with murine models but incubation temperature might be important, as *Drosophila* are incubated at 29°C, whereas mice and *G. mellonella* are maintained at 37°C. In addition *Drosophila* individuals are difficult to infect parenterally possibly leading to experimental inaccuracies.

Conclusion

Assessment of virulence following mutations in the *Aspergillus* genome has been pivotal in furthering our understanding of the pathogenesis of *Aspergillus* infections. There is significant interest in new classes of anti-infective agents that target virulence factors. The lure of potential novel drug targets has driven the need to screen mutants *in vivo*. Until recently the only suitable hosts to assess virulence were birds and mammals, the use of which raises ethical concerns. Recently Chamilos *et al*. suggested broadening the host range to include toll-deficient *D. melanogaster* [18]. They demonstrated that virulence assessed in *Drosophila* correlated with data generated in mice for many, but not all mutants.

This paper presents data on an alternative invertebrate host, *G. mellonella*, and demonstrates almost complete correlation of virulence when assessed in *G. mellonella* and mice.

Acknowledgements

We are thankful to Dr E. Bignell for the kind donation of the strains and The Fungal Research Trust for their funding.

Declaration of interest: The authors report no conflicts of interest. The authors are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 21 October 2010.