A cysteine/methionine auxotroph of the opportunistic fungus *Aspergillus flavus* is associated with host-range restriction: a model for emerging diseases

Lisa R. Scully and Michael J. Bidochka

Correspondence

Michael J. Bidochka
bidochka@brocku.ca

Department of Biological Sciences, 500 Glenridge Avenue, Brock University, St Catharines, Ontario, Canada L2S 3A1

Received 17 August 2005
Revised 21 October 2005
Accepted 24 October 2005

The evolution of host specialization in pathogens is a topic of considerable interest, particularly since it can represent a decisive step in the emergence of infectious diseases. *Aspergillus flavus* is an opportunistic fungus capable of infecting a wide variety of hosts, including plants, insects and mammals, although with low virulence. Here the derivation of an *A. flavus* strain that exhibits severe host restriction is reported. This strain exhibited a severe diminution or a complete lack of conidial production on a variety of standard agar media and on various plant species. However, it retained its ability to infect insects from various orders and to re-emerge from and adequately conidiate on the insect cadavers as a culmination of the pathogenic life cycle. This strain, demonstrating insect-dependent conidiation, was discovered to be a cysteine/methionine auxotroph due to an inability to reduce sulfate to sulfite. However, other *A. flavus* auxotrophs tested for plant and insect host range failed to show insect-dependent conidiation. An association between this specific auxotroph and a decreased host range is shown, emphasizing the role of nutrition in the host–pathogen relationship with respect to host restriction and evolution towards obligate pathogenesis.

INTRODUCTION

In terms of host–pathogen relationships, micro-organisms can be classified into one of three categories: opportunistic, facultative or obligate pathogens (Scheffer, 1991). Opportunistic pathogens persist on a wide range of organic substrates, but exhibit low virulence towards a broad array of living hosts, particularly if the host is injured or immunocompromised. Facultative pathogens efficiently colonize and produce disease in a narrow range of healthy hosts, although they are capable of surviving outside the host. Micro-organisms that require a living host for survival and replication constitute the obligate pathogens and generally have narrow host ranges. Specialized pathogens, such as the obligate and facultative pathogens, probably evolved from less specialized opportunistic pathogens, representing a theoretical framework for the emergence of infectious diseases and host specialization (Scheffer, 1991).

To study the evolution of host specificity and the development of infectious diseases, we utilized an insect–fungal model of infection involving the fungus *Aspergillus flavus* as the pathogen and wax moth larvae [*Galleria mellonella* (Lepidoptera)], as the insect host. *A. flavus* is an opportunistic fungal pathogen capable of thriving saprobially as well as infecting a wide variety of living hosts, including plants, insects and mammals, albeit with low virulence. *A. flavus* is an ideal model pathogen for the study of adaptation and host restriction, since it possesses a broad spectrum of protein- and polysaccharide-hydrolysing enzymes for exploiting available living and non-living organic resources (St Leger *et al.*, 2000). With respect to insect pathogenicity, *A. flavus* is an opportunistic pathogen of silkworms, grasshoppers, houseflies and mealy bugs among others (Gupta & Gopal, 2002). *A. flavus* is the causative agent of aspergillosis documented in the silkworm *Bombyx mori* (Kumar *et al.*, 2004). *A. flavus* infection of insects involves conidium germination on the insect surface, followed by a breach of the cuticle and access to the nutrient-rich haemocoel. Inside the infected insect, the hyphae multiply extensively, causing death and subsequent mycelial emergence from and mummification of the cadaver. The emergent mycelia conidiate on the surface of the cadaver, providing the transmissible propagules dispersed to other hosts for the

Abbreviations: AFLP, amplified fragment length polymorphism; Cz, Czapek solution Agar; NA, nutrient agar; PDA, Potato Dextrose Agar; YPD, yeast peptone dextrose agar.

The GenBank/EMBL/DDBJ accession number for the ITS region sequence reported in this paper is AY521473.

continuation of the pathogenic cycle (Kumar *et al.*, 2004). In the event that a suitable host is not available, the conidia germinate and grow saprobially.

In this study, we employed a serial propagation scheme involving the continuous passage of *A. flavus* through larvae of *G. mellonella*, mimicking the repeated infection and transmission of a disease-causing, opportunistic pathogen through a single species of a susceptible host. The result was a strain of *A. flavus* that exhibited a severe diminution in conidial production on standard artificial media, while retaining pathogenicity towards the insect host, including conidial production on the dead host during the final stage of pathogenesis. In addition, it demonstrated a reduction in host range as a plant pathogen compared to the original strain as well as an auxotrophic requirement for cysteine/methionine due to an inability to reduce sulfate to sulfite. In essence, this strain represents the progression by an opportunistic fungus towards obligate insect pathogenesis in that it can only efficiently complete its life cycle (i.e. mycelial growth followed by conidial production) as a consequence of infection of an insect host. In the opportunistic pathogen *A. flavus*, we show an association between auxotrophy and severe host restriction constituting a shift towards obligate pathogenesis that emphasizes the role of nutrition in evolving host–pathogen relationships.

METHODS

Fungi, insect and plant species. This study utilized *Aspergillus flavus* strains 6982, 4336, 9308 (University of Alberta Microfungus Collection and Herbarium), 1010, 2159 (Collection of Entomopathogenic Fungal Cultures USDA-ARS Plant Protection Research Unit), 46109 (*his*[−]), 46110 (*ile*[−] *val*[−]), 46111 (*pro*[−]), 46112 (*phe*[−]) and 46114 (*met*^{−1}) (American Type Culture Collection, Manassas, VA, USA) (Papa, 1980), *Aspergillus fumigatus* FR 2923-97 (Rosehart *et al.*, 2002) and *Emericella nidulans* 7677 (University of Alberta Microfungus Collection and Herbarium). All strains were maintained on Petri dishes containing 10 ml volumes of Potato Dextrose Agar (PDA) incubated at 30 °C for 7 days.

The following insect and plant species were used in this study: mealworms [*Tenebrio molitor* (Coleoptera)], blowfly larvae [*Sarcophaga bullata* (Diptera)] and crickets [*Acheta domestica* (Orthoptera)] from Ward's Scientific, wax moth larvae [*Galleria mellonella* (Lepidoptera)] from Peterborough Live Bait (Peterborough, ON, Canada) and milkweed bugs [*Oncopeltus fasciatus* (Hemiptera)] from Boreal, tomato (*Lycopersicon* sp.), corn (*Zea* sp.), green bean (*Phaseolus* sp.), pea (*Pisum* sp.) and cucumber (*Cucumis* sp.) from Zehrs (St Catharines, ON, Canada) and alfalfa [*Medicago* sp. (Boreal)].

Serial propagation of *A. flavus* through *G. mellonella* larvae. To propagate *A. flavus* 6982 through *G. mellonella*, 25 larvae were topically inoculated on the dorsal surface with 5 µl 1 × 10⁵ conidia ml^{−1} suspension, placed separately into clean plastic vials and incubated at 30 °C. At death, insects were washed for 1 min in 1% (v/v) NaOCl followed by two washings of 2 min each in sterile dH₂O. This procedure effectively removed surface conidia from insect cadavers, allowing only cuticle-breaching, pathogenic conidia to survive (results not shown). Emergent conidia from the first insect to die were harvested in 3 ml 0.01% (v/v) Triton X-100, 0.02% (w/v) chloramphenicol solution. After quantifying the suspension microscopically with a haemocytometer, 25 larvae were topically

inoculated to obtain the next generation of fungal passage through the insect host. Five replicate lineages of this scheme were performed, each consisting of six generations of fungal passage through the host. Fungal samples from each generation of each lineage were stored at −80 °C in 25% glycerol and subcultured onto PDA. The initial conidial suspension used in this scheme was mutagenized by exposure to UV-A light (400 µW cm^{−2}) for 3 min, resulting in 80% mortality of conidia.

From this artificial selection scheme, a single strain of *A. flavus* was derived that exhibited a drastic reduction in conidial production on PDA, while producing conidia on the infected insect cadaver as the culmination of the pathogenic cycle. This strain was designated *Af6982con*^{ins} because it demonstrated efficient conidial production on the infected insect cadaver, but not on PDA. *Af6982con*^{ins} demonstrated a similar colony phenotype when subcultured onto nutrient agar (NA; Difco) and yeast peptone dextrose agar (YPD; 0.2% yeast extract, 1% peptone, 2% glucose, 1.5% agar), but demonstrated no growth on Czapek solution agar (Cz; Difco).

DNA extraction, ITS amplification and amplified fragment length polymorphism (AFLP) analysis. To confirm that the derived strain was not a contaminant, the DNA was extracted using the DNeasy tissue extraction kit (Qiagen), and the ITS region was amplified and sequenced using standard protocols and ITS primers (White *et al.*, 1990).

To further confirm the relationship of the derived strain to the parental strain as well as to other strains and species of *Aspergillus*, we used AFLP analysis. Genomic DNA of *A. flavus*, *A. fumigatus* and *E. nidulans* isolates was prepared using the DNeasy tissue extraction kit (Qiagen). Five hundred nanograms of fungal DNA was digested with 1 U *Eco*RI and 1 U *Mse*I in a total volume of 10 µl, following the manufacturer's instructions (New England Biolabs). Following digestion, reactions were incubated at 70 °C for 15 min. Ligation was performed in a total volume of 20 µl by adding 2 pmol E adapter, 20 pmol M adapter (Table 1) and 0.4 U T4 DNA ligase (New England Biolabs) to the total volume of the digestion reaction. The ligation reactions were incubated at 20 °C for 2 h. The digestion–ligation products were diluted 1:10 with TE (10 mM Tris/HCl, 1 mM EDTA, pH 8.0).

Preamplification was carried out in a total volume of 30 µl, containing 5 µl diluted digestion–ligation product, 0.5 µM primer EO, 0.5 µM primer MO (Table 1) and 15 µl *Taq* PCR Master Mix (Qiagen). The PCR conditions were: 20 cycles of 94 °C for 30 s, 56 °C for 60 s and 72 °C for 60 s. The preamplification products were diluted 1:10 with TE.

Table 1. Adapters and primers used for AFLP analysis

Type	Name	Sequence (5'–3')
Adapter	E adapter	CTCGTAGACTGCGTACC AATTGGTACGCAGTCTAC
	M adapter	GACGATGAGTCCTGAG TACTCAGGACTCAT
Primer	EO	GACTGCGTACCAATTTC
	E + A	GACTGCGTACCAATTCA
	E + AG	GACTGCGTACCAATTCAG
	E + GC	GACTGCGTACCAATTCGC
	MO	GATGAGTCCTGAGTAA
	M + T	GATGAGTCCTGAGTAAT
	M + A	GATGAGTCCTGAGTAAA
	M + CAG	GATGAGTCCTGAGTAACAG

Eleven selective amplification reactions were performed using the following primer combinations: EO/M + CAG, E + A/M + A, E + A/M + CAG, E + GC/MO, E + GC/M + T, E + GC/M + A, E + GC/M + CAG, E + AG/MO, E + AG/M + T, E + AG/M + A and E + AG/M + CAG (Table 1). The selective amplification reactions contained 5 µl diluted preamplification product, 0.5 µM each primer and 11 µl *Taq* PCR Master Mix (Qiagen) in a total volume of 22 µl. The PCR conditions were: 6 cycles of 94 °C for 30 s, 65 °C for 30 s, 72 °C for 60 s; 6 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 60 s; 23 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s; followed by a final extension at 72 °C for 5 min.

The selective amplification products were analysed on 10% (19:1) denaturing polyacrylamide gels (Sequi-Gen GT Nucleic Acid Electrophoresis Cell; Bio-Rad), which were subsequently silver-stained (Blum *et al.*, 1987).

A similarity matrix using simple matching coefficients was constructed using NYSYS 2.1 after scoring the bands '1' for presence and '0' for absence. Cluster analysis was performed using the unweighted pair-group method using arithmetic means (UPGMA).

Insect and plant bioassay. Virulence towards *G. mellonella* larvae was determined by topical application of 25 larvae with $5 \mu\text{l } 1 \times 10^8$ conidia ml^{-1} and recording the percentage mortality at day 10. Fisher's exact tests or χ^2 tests were used to compare the percentage mortality of *A. flavus* strains to each other and to the control bioassay, which was topical application of 5 µl of 0.01% (v/v) Triton X-100. Pathogenicity tests against all insect species were performed by injecting conidial suspensions ($3 \mu\text{l } 1 \times 10^6$ conidia ml^{-1}) into the haemocoel using a clean syringe (gauge = 22). All insects were placed individually in clean plastic vials, incubated at 30 °C and monitored daily for mortality and subsequent conidiation on the cadaver. Inoculation via injection was utilized to ensure infection.

For plant pathogenicity tests, various plant species were washed in 1% NaOCl for 1 min followed by three 1 min washings in sterile dH₂O. Plants were inoculated by piercing the tissue with a sterilized needle, followed by inoculation of the wounded area with 2 µl of a 1×10^6 conidia ml^{-1} suspension. Some suspensions were supplemented with a 10 mM final concentration of either cysteine or methionine. The plants were incubated on 1% water agar at 30 °C for 7 days before assessing mycelial growth and conidiation.

For all plant and insect bioassays utilizing auxotrophic *A. flavus* strains, emergent conidia were tested to determine if they retained the auxotrophic phenotype. The conidia were subcultured onto Cz and Cz supplemented with 10 mM of the required amino acid. Lack of growth in the absence of supplementation confirmed the auxotrophic phenotype. Any plants or insects with conidia that failed to display an auxotrophic phenotype were omitted from the data since this indicated reversion.

Conidial counts. To quantify conidial production of a subculture grown on PDA with or without 10 mM cysteine or methionine, a 2.54 cm diameter agar plug was removed from halfway between the centre of the Petri dish and the perimeter. The plug was homogenized in 5 ml 0.01% Triton X-100 for 1 min in a 50 ml polystyrene tube with a motorized homogenizer (Greiner Scientific). Conidia were counted using a haemocytometer.

Quantification of conidia produced on *G. mellonella* and *Zea* sp. was performed by vortexing a conidiated insect cadaver or corn kernel in 3 ml 0.01% Triton X-100, 0.02% chloramphenicol. The resulting suspension was counted using a haemocytometer.

Microscopy. Samples of fungal material from *G. mellonella* larvae and PDA plates were stained with lactophenol cotton blue and examined under a bright-field microscope.

Detection of auxotroph phenotype and reversion. Wells of 96-well plates were filled with 200 µl Cz and individually supplemented with 1 µmol of one of the following amino acids: L-α alanine, L-arginine, L-asparagine, L-aspartic acid, L-cysteine, L-glutamine, L-glutamic acid, glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-phenylalanine, L-proline, L-serine, L-threonine, L-tryptophan, L-tyrosine and L-valine. Wells were inoculated with 5 µl of a 1×10^6 conidia ml^{-1} suspension.

Screening for auxotrophic reversion was performed by plating 50 µl 1×10^6 conidia ml^{-1} of *Af6982con^{ins}* suspension onto each of 40 Cz Petri dishes and observing fungal growth.

Sulfur assimilation enzyme assays. Flasks containing 100 ml YPD broth (0.2% yeast extract, 1% peptone, 2% glucose) were inoculated with 50 µl 1×10^6 conidia ml^{-1} and incubated at 27 °C shaking at 250 r.p.m. for 3 days. Fungi were then filtered from the broth, washed with Czapek–Dox broth (Difco) and transferred to flasks containing 100 ml Czapek–Dox broth incubated for 2 days at 27 °C and 250 r.p.m. After filtering from the medium, the fungi were crushed in liquid nitrogen and the resulting material was used to assay the activity of ATP sulfurylase as described by Segel *et al.* (1987), the conversion of sulfate to sulfite (Breton & Surdin-Kerjan, 1977) and sulfite reductase (De Vito & Dreyfuss, 1964). Sulfate permease function was assessed based on the inhibition of growth by the toxic sulfate analogue chromate on Cz agar containing 10 mM methionine and 10 mM potassium chromate as described by Arst (1968).

RESULTS

A strain of *A. flavus* demonstrating insect-dependent conidiation

A. flavus was subjected to serial propagation through *G. mellonella* larvae for six generations. During serial passage, one of the lineages produced a strain that demonstrated a remarkable characteristic. Although this strain remained pathogenic towards the larvae, producing conidia on the surface of the insect cadaver as the final stage of pathogenesis, it demonstrated a drastic reduction in conidial production when grown on PDA (Fig. 1). A similar reduction was observed on a variety of other agar media, namely YPD and NA (data not shown), and unlike the original strain, no growth occurred on Cz. Conidial production exhibited by this strain when grown on PDA was 75-fold less than that of the original strain previous to passage through the insect host (Fig. 2). Conversely, the amount of conidia produced by this strain on the insect cadaver at the completion of the pathogenic cycle was much higher, exhibiting only a 10-fold reduction compared to the parental strain by either topical application or injection (Fig. 2). These characteristics were displayed by this strain at each of generations 2–6 of passage through the insect (data not shown).

The BLAST search results of the ITS sequence of the selected strain exhibited a 97% similarity with the ITS sequences of other *A. flavus* strains (GenBank accession no. AY521473).

Fig. 1. Conidiation of *Af6982* and *Af6982con*^{ins} on *G. mellonella* larvae (injected with $3 \mu\text{l } 1 \times 10^6$ conidia ml^{-1}) and PDA. Top row, mycelial emergence and conidiation on larvae by (a) *Af6982* and (b) *Af6982con*^{ins}; second row, microscopic examination of (c) *Af6982* and (d) *Af6982con*^{ins} from infected larvae; third row, mycelial growth and conidiation on PDA by (e) *Af6982* and (f) *Af6982con*^{ins}; bottom row, microscopic examination of (g) *Af6982* and (h) *Af6982con*^{ins} from PDA.

Because of its dependency on the insect host for adequate conidial production, this strain is designated *Af6982con*^{ins}.

Virulence and host range of *Af6982con*^{ins}

The virulence of *Af6982con*^{ins} towards *G. mellonella* larvae (64 % mortality at day 10) remained comparable to that of *Af6982* (48 % mortality at day 10, $P=0.39$; Fisher's exact test) and statistically significant compared to a control inoculation ($P=0.001$ and 0.03 , respectively; Fisher's exact test) during topical application bioassays ($5 \mu\text{l } 1 \times 10^8$ conidia ml^{-1}).

Fig. 2. Relative difference in number of conidia produced by *Af6982* and *Af6982con*^{ins} (hatched bars) or *Af46114* (*met*⁻) (black bars) on PDA, corn and *G. mellonella* larvae. The relative difference was calculated as the number of conidia produced by *Af6982* divided by the number of conidia produced by *Af6982con*^{ins} or *Af46114*. PDA and corn were supplemented with 10 mM of either cysteine (Cys) or methionine (Met). The amount of conidia on *G. mellonella* larvae was assessed after both topical application ($5 \mu\text{l } 1 \times 10^8$ conidia ml^{-1}) and injection ($3 \mu\text{l } 1 \times 10^6$ conidia ml^{-1}). For all substrates, the amount of conidia was calculated as the mean of three replicates.

Insects of various orders as well as a range of plant species were inoculated with *Af6982* and *Af6982con*^{ins} to determine host ranges established by the essential pathogenic property of conidiation on the infected host, indicative of successful colonization. The results indicated that *Af6982con*^{ins} and its parental strain *Af6982* both possessed the ability to conidiate on a variety of infected insects (injected with $3 \mu\text{l } 1 \times 10^6$ conidia ml^{-1}). However, unlike strain *Af6982*, *Af6982con*^{ins} failed to produce lesions or exhibit any growth on the infected plant species except for the corn kernels on which it produced some mycelia and exceptionally few conidia (Table 2, Fig. 2).

Amino acid supplementation of *Af6982con*^{ins}

Af6982con^{ins} was grown on Cz supplemented with single amino acids to detect an auxotrophic phenotype. Whereas the parental strain exhibited growth regardless of amino acid supplementation, *Af6982con*^{ins} showed no growth on Cz except when supplemented with either cysteine or methionine (data not shown).

Although supplementation of *Af6982con*^{ins} with either cysteine or methionine failed to restore pathogenicity

Table 2. Conidiation of *A. flavus* strains *Af6982*, *Af6982con^{ins}*, the revertant and *Af46114* (*met⁻*) on insects of various orders (injected with $3 \mu\text{l } 1 \times 10^6$ conidia ml^{-1}) and a range of plant species

Data are representative of at least two independent trials (+++, much conidiation; ++, moderate conidiation; +, little conidiation; –, no conidiation).

Kingdom	Order	Common name	Conidiation*			
			<i>Af6982</i>	<i>Af6982con^{ins}</i>	Revertant	<i>Af46114</i>
Animalia	Coleoptera	Mealworm	+++	++	+++	+++
Animalia	Diptera	Blowfly larvae	++	+	++	++
Animalia	Lepidoptera	Wax moth larvae	+++	++	+++	+++
Animalia	Hemiptera	Milkweed bug				
Animalia	Orthoptera	Crickets	–	–	–	–
Plantae	Solanales	Tomato	+++	–	+++	+
Plantae	Poales	Corn	+++	+	+++	+++
Plantae	Fabales	Alfalfa	++	–	++	++
Plantae	Fabales	Pea	++	–	++	+
Plantae	Fabales	Green bean	++	–	++	–
Plantae	Cucurbitales	Cucumber	+	–	+	–

*Note: here we indicate that conidiation is the culmination of mycelial growth.

Fig. 3. Functionality of sulfur assimilation pathway enzymes in *Af6982* (wt), *Af6982con^{ins}* (*con^{ins}*) and *Af46114* (*met⁻*). (a) Sulfur assimilation pathway; (1) sulfate permeases, (2) ATP sulfurylase, (3) adenosine 5'-phosphosulfate (APS) kinase, (4) 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, (5) sulfite reductase (modified from Marzluf, 1997). (b) Growth in the presence (left) and absence (right) of $\text{K}_2\text{Cr}_2\text{O}_7$. (c) ATP sulfurylase activity. (d) Sulfate to sulfite reduction activity. (e) Sulfite reductase activity. ND represents no enzyme activity detected. Values are the means of three independent trials.

towards most plant hosts, on PDA and corn conidial production relative to *Af*6982 was brought to a level similar to that generated on the insect host (Fig. 2).

Sulfur assimilation enzyme activity of *Af*6982con^{ins}

The ability of *Af*6982con^{ins} to import sulfate and convert it to cysteine or methionine was assessed by analysing the functionality of the enzymes of the sulfur assimilation pathway (Fig. 3a). Both *Af*6982 and *Af*6982con^{ins} failed to grow in the presence of chromate, a toxic sulfate analogue, indicating a functional sulfate permease (Fig. 3b). While both *Af*6982 and *Af*6982con^{ins} demonstrated comparable levels of ATP sulfurylase (Fig. 3c, $t=0.74$, $P=0.26$) and sulfite reductase (Fig. 3e, $t=0.13$, $P=0.46$) activity, *Af*6982con^{ins} failed to show any sulfate to sulfite reduction activity (Fig. 3d).

Spontaneous reversion

Screening for spontaneous reversion of *Af*6982con^{ins} by plating conidia on Cz produced one revertant in 2×10^6 conidia that exhibited full recovery of the growth and conidiation properties of the parent strain when grown on PDA, Cz, NA and YPD. The revertant also demonstrated full recovery of the conidiation and pathogenicity properties of the parental strain *Af*6982 when assayed against various plant species, while maintaining pathogenicity towards insects (Table 2). In addition, the revertant retained a level of virulence towards *G. mellonella* larvae that was statistically different from a control inoculation (32 % mortality by day 10, $P=0.02$; Fisher's exact test) and statistically equivalent to that of the original strain *Af*6982 and *Af*6982con^{ins} ($P=0.08$; χ^2 test).

AFLP analysis

Cluster analysis based on the presence or absence of AFLP bands (Fig. 4) indicated that isolates of the original strain *Af*6982, the strain demonstrating insect-dependent conidiation *Af*6982con^{ins} and the revertant possessed 99 % similarity by simple matching coefficients. The similarity of these isolates to other *A. flavus* strains ranged from 57 to 94 %, while their similarity to *A. fumigatus* and *E. nidulans* was 49 and 47 %, respectively. Although differences were observed between the isolates of *Af*6982, *Af*6982con^{ins} and the revertant, there were no diagnostic polymorphisms that distinguished the isolates of the original strain from those of the strain demonstrating insect-dependent conidiation.

Virulence and host range of *A. flavus* auxotrophs

Five *A. flavus* auxotrophs with requirements for histidine, proline, isoleucine and valine, phenylalanine or methionine (Papa, 1980; and confirmed in this study) all exhibited minimal to no growth on Cz. All five auxotrophs exhibited infection and substantial conidiation on *G. mellonella* cadavers when injected or applied topically to the cuticle. Three of the five auxotrophic strains demonstrated a statistically significant percentage mortality (Table 3) compared to a control inoculation during topical application bioassays ($5 \mu\text{l } 1 \times 10^8$ conidia ml^{-1}). However, unlike *Af*6982con^{ins}, most of these auxotrophs retained the ability to infect alfalfa (Table 3). Further investigation of *A. flavus* 46114, which demonstrated an auxotrophic requirement for methionine similar to that of *Af*6982con^{ins}, showed an ability to infect a variety of both plants and insects (Table 2) and exhibited no diminution in conidial production on PDA, corn or *G. mellonella* larvae relative to *Af*6982 (Fig. 2). As with *Af*6982, *Af*46114 failed to grow in the presence of chromate (Fig. 3b), demonstrating a functional sulfate permease,

Fig. 4. UPGMA cluster analysis of *A. fumigatus*, *E. nidulans* and single or multiple isolates of *A. flavus* based on simple matching coefficients generated from the presence or absence of AFLP bands. The number after the strain number refers to one of the single colony isolates of *Af*6982, *Af*6982con^{ins} or the revertant.

Table 3. Conidiation properties and virulence of *A. flavus* auxotrophs with respect to alfalfa (*Medicago* sp.) and *G. mellonella* hosts

Virulence was determined as the percentage mortality by day 10 after topical application of $5 \mu\text{l } 1 \times 10^8$ conidia ml^{-1} (+, conidiation; –, no conidiation).

Strain	Phenotype	Conidiation on alfalfa	Conidiation on <i>G. mellonella</i>		Virulence towards <i>G. mellonella</i>	
			Injection	Top. appl.	Mortality (%)	<i>P</i> value
Af6982	Wild-type	+	+	+	48*	0.03*
Af6982con ^{ins}	<i>cys/met</i> [–]	–	+	+	64*	0.001*
Revertant	Wild-type	+	+	+	32*	0.02*
Af46109	<i>his</i> [–]	–	+	+	24	0.72
Af46110	<i>ile</i> [–] <i>val</i> [–]	+	+	+	12	0.99
Af46111	<i>pro</i> [–]	+	+	+	56*	0.007*
Af46112	<i>phe</i> [–]	+	+	+	60*	0.003*
Af46114	<i>met</i> [–]	+	+	+	53*	0.012*

*Represents statistically significant percentage mortality compared to control inoculation (topical application of $5 \mu\text{l } 0.01\%$ Triton X-100) using Fisher's exact test.

and exhibited comparable levels of ATP sulfurylase (Fig. 3c, $t=0.01$, $P=0.50$), sulfate to sulfite reduction (Fig. 3d, $t=0.66$, $P=0.28$) and sulfite reductase activity (Fig. 3e, $t=0.56$, $P=0.30$).

DISCUSSION

This study reports the derivation of a strain of the opportunistic fungus *A. flavus* that exhibited characteristics similar to those of obligate pathogens. The properties displayed by this strain, designated Af6982con^{ins}, include a drastic reduction in its ability to produce conidia on artificial media paralleled by adequate conidiation as the end result of the pathogenic cycle when restricted to the insect host. In addition to its decreased saprobic abilities, Af6982con^{ins} also demonstrated a decreased host range compared to the original strain Af6982. Whereas Af6982 infected, produced mycelia and generated conidia on insects from different orders and various plant species, Af6982con^{ins} generated conidia most efficiently on insects, while failing to produce mycelia and conidia on most plant species. Furthermore, Af6982con^{ins} demonstrated these properties while maintaining virulence towards *G. mellonella* larvae at a level comparable to that of the original strain Af6982. Thus, while retaining pathogenicity towards insects, Af6982con^{ins} demonstrated a dependency on the insect host as its most robust option for reproduction as the culmination of its pathogenic cycle. Unlike Af6982, neither growth on standard artificial media nor reproduction as a plant pathogen represented a suitable option. These characteristics of Af6982con^{ins} constitute a shift towards obligate insect pathogenesis.

Supplementation of Cz with various amino acids revealed that Af6982con^{ins} was a cysteine/methionine auxotroph. This nutrient deficiency provides a plausible explanation for

its dependency on the insect host for reproduction. Such nutrient requirements suggest that infection and propagation through the larval host allowed this auxotroph to grow and conidiate as it gained the required amino acids from the host, most likely from the protein- and amino-acid-rich insect cuticle or haemolymph (Hackman & Goldberg, 1976; Paterson *et al.*, 1994; Hanzal & Jegorov, 1991). However, neither standard artificial media nor plant species were able to provide Af6982con^{ins} with the nutrients required for adequate conidiation. Accordingly, Af6982con^{ins} exhibited properties similar to those of obligate pathogens because it was severely impeded in its ability to complete its lifecycle via the production of conidia unless it was allowed to infect, colonize, gain nutrients from and finally re-emerge from the insect to conidiate on the cadaver surface.

Many pathogens designated obligate can proliferate on artificial media when they are provided with the appropriate nutrients. For example, the obligate mammalian pathogens *Mycobacterium tuberculosis* and *Yersinia pestis* can be cultured on Lowenstein–Jensen medium and TMH medium, respectively (Tanoue *et al.*, 2002; Qiu *et al.*, 2005). *Cronartium quercuum*, the obligate fungal pathogen of oak tree leaves, can be cultivated on PGY medium (Warren & Covert, 2004). In principle, the cysteine/methionine auxotroph described in this study behaves no differently. Besides demonstrating a restricted host range, Af6982con^{ins} requires enhanced artificial media for culture growth. These characteristics show similarity with other obligate pathogens and demonstrate an evolution towards obligate pathogenesis.

Unlike Af6982con^{ins}, a variety of other *A. flavus* auxotrophs demonstrating nutrient deficiencies did not show a diminished host range. Four of the five auxotrophs demonstrated mycelial growth and conidiation on the insect host *G. mellonella* larvae as well as the ability to infect and conidiate

on alfalfa leaves. Specifically, *A. flavus* strain 46114, which displayed an auxotrophic requirement for methionine similar to that of *Af6982con^{ins}*, demonstrated that its nutrient deficiency neither reduced the host range of *Af46114* nor hindered its ability to produce conidia on a variety of substrates. Enzyme assays revealed that unlike *Af46114*, *Af6982con^{ins}* is deficient in the ability to reduce sulfate to sulfite, indicating that the metabolic cause of auxotrophy in these two strains is different. Such metabolic differences are a probable explanation for the observed differences in host range.

Of the 20 essential amino acids, cysteine and methionine are the only two that contain sulfur. Not surprisingly, their biosynthetic and metabolic pathways are highly interconnected (Ono *et al.*, 1999), leading to the hypothesis that the cysteine/methionine auxotrophy of *Af6982con^{ins}* was a result of a single-gene mutation in a common biosynthetic pathway. We obtained a spontaneous revertant of *Af6982con^{ins}* that regained the characteristics of the original opportunistic pathogen *Af6982*, namely conidiation on artificial media and plant species while preserving insect pathogenicity and a similar level of virulence towards *G. mellonella* larvae. The recovery of a spontaneous revertant implied that the obligate pathogen characteristics of *Af6982con^{ins}* were due to a single gene mutation, since the probability of obtaining a spontaneous revertant after multiple mutations was unlikely.

Because fungi are able to interconvert cysteine and methionine (Ono *et al.*, 1999), a single gene mutation resulting in auxotrophy that is recovered by either cysteine or methionine implies a mutation in the sulfur assimilation pathway essential for the synthesis of either amino acid (Marzluf, 1997). Indeed, we showed that *Af6982con^{ins}* was unable to reduce sulfate to sulfite. We hypothesize that the auxotrophy

of *Af6982con^{ins}* is due to an incapacitating mutation in one of the enzymes essential for the reduction of sulfate to sulfite, presumably either adenosine 5'-phosphosulfate (APS) kinase or 3'-phosphoadenosine 5'-phosphosulfate (PAPS) reductase, since ATP sulfurylase activity is detected in *Af6982con^{ins}*. Further experiments are under way to genetically characterize this mutation.

The high level of similarity between the original opportunistic strain *Af6982*, the strain demonstrating insect-dependent conidiation *Af6982con^{ins}* and the revertant indicated by the AFLP cluster analysis suggested that they are derived from the same strain (Savelkoul *et al.*, 1999; Rademaker *et al.*, 2000; Janssen *et al.*, 1997). In all likelihood, a mutant produced during the UV mutagenesis of the initial conidial suspension was selected during passage through the insect that resulted in the derivation of a strain, which displayed insect-dependent conidiation. Subsequent reversion of the mutation allowed for recovery of the revertant. Although a small number of differences was observed among the isolates of the *Af6982*, *Af6982con^{ins}* and the revertant, this degree of dissimilarity has been observed between multiple isolates of a single strain of other asexually reproducing microbes (Savelkoul *et al.*, 1999; Rademaker *et al.*, 2000; Janssen *et al.*, 1997). These differences may be due to noise or hysteresis in this PCR-based technique. Alternatively, they may be legitimately due to mutations as asexually reproducing microbes are not genetically static during subculturing.

Our results indicated an association between a single gene mutation resulting in auxotrophy and host restriction, in turn resulting in evolution towards obligate insect pathogenesis by an opportunistic pathogen. As shown in Fig. 5, such a mutation may represent a decisive step in the emergence of a highly virulent obligate pathogen as it may

Fig. 5. A hypothetical model to explain host restriction of an opportunistic pathogen through to host specialization and increased virulence as an obligate pathogen. The opportunistic pathogen exhibits a broad host-range but relatively low virulence. The obligate pathogen exhibits a narrow host-range but relatively higher virulence. (a) Host restriction associated with a single gene mutation and auxotrophy. (b) Spontaneous reversion of that mutation restores the broad host-range. (c) Restricted host-range applies selection pressure to the pathogen, resulting in the evolution of host specialization and increased virulence.

provide selection pressure for further adaptation towards increased virulence. This hypothesis recognizes that an essential prerequisite to pathogenicity is the capacity of the host to provide suitable nutrients for the pathogen. The host must satisfy the nutritional requirements of the pathogen for the latter to proliferate and cause disease (Garber, 1956). Recent reviews highlight the importance of host–microbe interactions in the production of tissue damage and disease, recognizing that host nutritional status and the ability of a microbe to survive and replicate in a given host are important aspects of pathogenicity (Casadevall & Pirofski, 1999, 2001).

In many cases, the host is capable of providing nutrients for the growth of an auxotrophic strain of a pathogen such that the microbe retains its pathogenicity and does not exhibit a decrease in virulence. For example, biochemical mutants of *Klebsiella pneumoniae* deficient in the synthesis of one amino acid, threonine, tyrosine, methionine, leucine or histidine, were as virulent as the wild-type towards mice (Garber *et al.*, 1952). The facultative bacterial pathogen *Listeria monocytogenes* auxotrophic for uracil, phenylalanine, glycine or proline displayed virulence and growth rates similar to the parental strain when bioassayed against mice (Marquis *et al.*, 1993). Similarly, cysteine or methionine auxotrophs of a variety of pathogens retain pathogenicity towards their hosts. Methionine auxotrophs of *Pseudomonas syringae* pv. *glycinea* retain pathogenicity towards soybeans (Thomas & Leary, 1980), and methionine or cysteine-methionine auxotrophs of *Candida albicans* show no decrease in virulence towards mice (Manning *et al.*, 1984). With respect to survival in mice, methionine auxotrophs of *Mycobacterium bovis* show no attenuation compared to the wild-type (Wooff *et al.*, 2002).

It follows from this explanation of the role of nutrition in pathogenicity that host specificity of obligate pathogens may exist in part because the host is a source of adequate nutritional supplementation (Garber, 1960). Indeed, many obligate pathogens exhibit very specific nutrient requirements that must be supplied by the host for pathogenicity to occur, including the completion of the pathogen life cycle. *Rickettsia prowazekii*, the causative agent of louse-borne typhus, requires serine, glycine and proline from the host (Austin *et al.*, 1987; Austin & Winkler, 1988), while *Eubacterium suis*, an obligate bacterial pig pathogen causing pyelonephritis and cystitis, utilizes a multitude of carbohydrates, peptides and vitamins from its host (Wegienek & Reddy, 1982). *Yersinia pestis*, the causative agent of plague, possesses a phenotype devoid of the ability to synthesize many vitamins, amino acids and enzymes. As a consequence, *Y. pestis* cannot survive outside its mammalian or flea hosts. Comparison of the *Y. pestis* genome with that of a closely related, free-living, facultative pathogen, *Yersinia pseudotuberculosis*, implies that their divergence involved host-range restriction accompanied by metabolic gene inactivation in *Y. pestis* (Hinnebusch, 1997).

These studies indicate that at least part of the adaptation and restriction to a specific host of an obligate pathogen is correlated with loss of a biosynthetic component concomitantly supplemented by the host. The derivation of the auxotrophic strain Af6982con^{ins} in this study also implies a direct association between loss of a biosynthetic component and host restriction. Furthermore, this may be the first step in the emergence of infectious diseases because restriction of the pathogen to the host provides the opportunity for further adaptation via selection, involving the acquisition of virulence factors by spontaneous mutation, recombination or horizontal transmission.

ACKNOWLEDGEMENTS

This research was supported by a discovery grant from the National Sciences and Engineering Research Council of Canada to (NSERC) to M. J. B. and an NSERC postgraduate scholarship to L. R. S.

REFERENCES

- Arst, H. N. (1968). Genetic analysis of the first steps of sulphate metabolism in *Aspergillus nidulans*. *Nature* **219**, 268–270.
- Austin, F. E. & Winkler, H. H. (1988). Proline incorporation into protein by *Rickettsia prowazekii* during growth in Chinese hamster ovary (CHO-K1) cells. *Infect Immun* **56**, 3167–3172.
- Austin, R. E., Turco, J. & Winkler, H. H. (1987). *Rickettsia prowazekii* requires host cell serine and glycine for growth. *Infect Immun* **55**, 240–244.
- Blum, H., Beier, H. & Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.
- Breton, A. & Surdin-Kerjan, Y. (1977). Sulfate uptake in *Saccharomyces cerevisiae*: biochemical and genetic study. *J Bacteriol* **132**, 224–232.
- Casadevall, A. & Pirofski, L. (1999). Host–pathogen interactions: redefining the basic concepts of virulence and pathogenicity. *Infect Immun* **67**, 3703–3713.
- Casadevall, A. & Pirofski, L. (2001). Host–pathogen interactions: the attributes of virulence. *J Infect Dis* **184**, 337–344.
- De Vito, P. C. & Dreyfuss, J. (1964). Metabolic regulation of adenosine triphosphate sulfurylase in yeast. *J Bacteriol* **88**, 1341–1348.
- Garber, E. D. (1956). A nutrition-inhibition hypothesis of pathogenicity. *Am Nat* **90**, 183–193.
- Garber, E. D. (1960). The host as a growth medium. *Ann N Y Acad Sci* **88**, 1187–1194.
- Garber, E. D., Hackett, A. J. & Franklin, R. (1952). The virulence of biochemical mutants of *Klebsiella pneumoniae*. *Proc Natl Acad Sci U S A* **38**, 693–697.
- Gupta, A. & Gopal, M. (2002). Aflatoxin production by *Aspergillus flavus* isolates pathogenic to coconut insect pests. *World J Microbiol Biotechnol* **18**, 325–331.
- Hackman, R. H. & Goldberg, M. (1976). Comparative chemistry of arthropod cuticular proteins. *Comp Biochem Physiol* **55B**, 201–206.
- Hanzal, R. & Jegorov, A. (1991). Changes in free amino acid composition in haemolymph of larvae of the wax moth, *Galleria mellonella* L., during cold acclimation. *Comp Biochem Physiol* **100A**, 957–962.
- Hinnebusch, B. J. (1997). Bubonic plague: a molecular genetic case history of the emergence of an infectious disease. *J Mol Med* **75**, 645–652.

- Janssen, P., Maquelin, K., Coopman, R., Tjernberg, I., Bouvet, P., Kersters, K. & Dijkshoorn, L. (1997). Discrimination of *Acinetobacter* genomic species by AFLP fingerprinting. *Int J Syst Bacteriol* **47**, 1179–1187.
- Kumar, V., Singh, G. P. & Babu, A. M. (2004). Surface ultrastructural studies on the germination, penetration and conidial development of *Aspergillus flavus* Link: Fries infecting silkworm, *Bombyx mori* Linn. *Mycopathologia* **157**, 127–135.
- Manning, M., Snoddy, C. B. & Fromtling, R. A. (1984). Comparative pathogenicity of auxotrophic mutants of *Candida albicans*. *Can J Microbiol* **30**, 31–35.
- Marquis, H., Bouwer, H. G. A., Hinrichs, D. J. & Portnoy, D. A. (1993). Intracytoplasmic growth and virulence of *Listeria monocytogenes* auxotrophic mutants. *Infect Immun* **61**, 3756–3760.
- Marzluf, G. A. (1997). Molecular genetics of sulfur assimilation in filamentous fungi and yeast. *Annu Rev Microbiol* **51**, 73–96.
- Ono, B., Hazu, T., Yoshida, S., Kawato, T., Shinoda, S., Brzwczy, J. & Paszewski, A. (1999). Cysteine biosynthesis in *Saccharomyces cerevisiae*: a new outlook on pathway and regulation. *Yeast* **15**, 1365–1375.
- Papa, K. E. (1980). Dominant aflatoxin mutant of *Aspergillus flavus*. *J Gen Microbiol* **118**, 279–282.
- Paterson, J. C., Charnley, A. K., Cooper, R. M. & Clarkson, J. M. (1994). Partial characterization of specific inducers of a cuticle-degrading protease from the insect pathogenic fungus *Metarhizium anisopliae*. *Microbiology* **140**, 3153–3159.
- Qiu, J., Zhou, D., Han, Y. & 11 other authors (2005). Global gene expression profile of *Yersinia pestis* induced by streptomycin. *FEMS Microbiol Lett* **243**, 489–496.
- Rademaker, J. L. W., Hoste, B., Louws, F. J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P. & de Bruijn, F. J. (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. *Int J Syst Evol Microbiol* **50**, 665–677.
- Rosehart, K., Richards, M. H. & Bidochka, M. J. (2002). Microsatellite analysis of environmental and clinical isolates of the opportunist fungal pathogen *Aspergillus fumigatus*. *J Med Microbiol* **51**, 1128–1134.
- Savelkoul, P. H. M., Aarts, H. J. M., de Haas, J., Dijkshoorn, L., Duim, B., Otsen, M., Rademaker, J. L. W., Schouls, L. & Lenstra, J. A. (1999). Amplified-fragment length polymorphism analysis: the state of an art. *J Clin Microbiol* **37**, 3083–3091.
- Scheffer, R. P. (1991). Role of toxins in evolution and ecology of plant pathogenic fungi. *Experientia* **47**, 804–811.
- Segel, I. H., Renosto, F. & Seubert, P. A. (1987). Sulfate-activating enzymes. *Methods Enzymol* **143**, pp. 334–349. Edited by S. P. Colowick, N. O. Kaplan, W. B. Jakoby & O. W. Griffith. Orlando, FL: Academic Press.
- St Leger, R. J., Screen, S. E. & Shams-Pirzadeh, B. (2000). Lack of host specialization in *Aspergillus flavus*. *Appl Environ Microbiol* **66**, 320–324.
- Tanoue, S., Mitarai, S. & Shishido, H. (2002). Comparative study on the use of solid media: Lowenstein–Jensen and Ogawa in the determination of anti-tuberculosis drug susceptibility. *Tuberculosis* **82**, 63–67.
- Thomas, M. D. & Leary, J. V. (1980). Mutagenicity and pathogenicity in *Pseudomonas syringae* pv. *Glycinea* (*Pseudomonas glycinea*). *J Gen Microbiol* **121**, 349–355.
- Warren, J. M. & Covert, S. F. (2004). Differential expression of pine and *Cronartium quercuum* f. sp. *fusiforme* genes in fusiform rust galls. *Appl Environ Microbiol* **70**, 441–451.
- Wegienek, J. & Reddy, C. A. (1982). Nutritional and metabolic features of *Eubacterium suis*. *J Clin Microbiol* **15**, 895–901.
- White, T. J., Bruns, T., Lee, S. & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In *PCR Protocols: a Guide to Methods and Applications*, pp. 315–322. Edited by M. A. Innis, G. H. Gelfand, J. J. Sninsky & T. J. White. San Diego, CA: Academic Press.
- Wooff, E., Michell, S. L., Gordon, S. V., Chambers, M. A., Bardarov, S., Jacobs, W. R., Jr, Hewinson, R. G. & Wheeler, P. R. (2002). Functional genomics reveals the sole sulphate transporter of the *Mycobacterium tuberculosis* complex and its relevance to the acquisition of sulphur *in vivo*. *Mol Microbiol* **43**, 653–663.