Siderophore Biosynthesis But Not Reductive Iron Assimilation Is Essential for Aspergillus fumigatus Virulence

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Abstract

The ability to acquire iron in vivo is essential for most microbial pathogens. Here we show that Aspergillus fumigatus does not have specific mechanisms for the utilization of host iron sources. However, it does have functional siderophore-assisted iron mobilization and reductive iron assimilation systems, both of which are induced upon iron deprivation. Abrogation of reductive iron assimilation, by inactivation of the high affinity iron permease (FtrA), has no effect on virulence in a murine model of invasive aspergillosis. In striking contrast, A. fumigatus l-ornithine-N3-monoxygenase (SidA), which catalyses the first committed step of hydroxamate-type siderophore biosynthesis, is absolutely essential for virulence. Thus, A. fumigatus SidA is an essential virulence attribute. Combined with the absence of a sidA ortholog—and the fungal siderophore system in general—in mammals, these data demonstrate that the siderophore biosynthetic pathway represents a promising new target for the development of antifungal therapies.

Key words: fungal pathogenicity • aspergillosis • iron uptake • virulence factor • ornithine monoxygenase

Introduction

Aspergillus fumigatus is the most common airborne fungal pathogen of humans, causing life-threatening invasive disease in immunocompromised patients. The limitations of therapeutic intervention are reflected in mortality rates, dependent primarily on the immune status of the host, of up to 90% (1, 2). The importance of the host immune status has been underlined by the lack of identified specific virulence attributes in A. fumigatus (1, 3–6). Limiting access to essential nutrients is an often overlooked aspect of innate immunity (7). Iron is essential for most organisms, suggesting that its acquisition in vivo may be required for A. fumigatus to cause disease (8–10). Iron uptake systems are often required for bacterial and yeast virulence (11, 12).

Fungi have evolved various strategies, often used in parallel, to acquire iron. These include two high affinity uptake mechanisms, reductive iron assimilation, and siderophore-assisted iron mobilization (8–10). Siderophores are low molecular mass, organic, ferric iron–specific chelators, which are excreted during iron starvation (8, 9). Siderophore-bound iron is recovered by cells either by the reductive system or by specific transporters able to internalize the siderophore–iron complex. Furthermore, most fungi utilize intracellular siderophores as an iron storage compound. In Candida albicans, the siderophore transporter Arn1p/Sit1p is required for epithelial invasion and penetration but not for systemic infection (13). In systemic virulence of this fungus, the high affinity iron permease Ftr1, a component of the reductive iron assimilation system, has been shown to be essential (11), and high affinity heme uptake may play a role in virulence (14, 15). In contrast to bacteria (12), direct proof of the involvement of siderophore production in systemic fungal virulence is lacking. Indeed specific siderophore production was not found to be critical for phytopathogenesis of the basidiomycete Ustilago maydis (16). However, recent data indicate that A. fumigatus survival in human serum in vitro involves siderophore-mediated removal of iron from transferrin (17).

We have shown that l-ornithine-N3-monoxygenase catalyzes the first committed step in biosynthesis of both tricatetlylsuliramine C (TAFC) and desferriferricrocin (DFFC), the two major siderophores of Aspergillus nidulans (18). TAFC is a cyclic tripeptide consisting of three N2-acetyl-N3-cis-anhydrolevalonyl-N5-hydroxymyriste residues linked by ester bonds, and DFFC is a cyclic hexapeptide with the...
structure Gly-Ser-Gly-(N³-acetyl-N⁵-hydroxymornithine), (8). Linking of the hydroxamate groups and amino acid residues for formation of TAF/C and DFFC is performed by different nonribosomal peptide synthetases (18, 19) and so far only that required for synthesis of ferricrocin (FC) has been identified. Notably, the gene encoding l-ornithine-N⁵-monoxygenase is not clustered with other genes involved in siderophore biosynthesis in A. nidulans.

This report demonstrates that A. fumigatus employs three iron uptake mechanisms: ferrous iron uptake, reductive iron assimilation, and siderophore-mediated iron uptake. Siderophore-mediated iron uptake but not reductive iron assimilation was found to be essential for virulence in a murine model of invasive aspergillosis.

Materials and Methods

Growth Conditions, Analysis of Siderophore Production, and Up-take. Generally, A. fumigatus strains were grown at 37°C in minimal medium (+Fe-Aspergillus minimal medium (AMM), iron-replete conditions) according to Pontecorvo et al. (20) containing 1% (wt/vol) glucose as the carbon source, 20 mM glutamine as the nitrogen source, and 10 μM FeSO₄. For iron-depleted conditions, iron was omitted. The blood agar was –Fe-AMM conditions, iron was omitted. The blood agar was –Fe-AMM containing 1.5 mM FeSO₄ and the nitrogen source, and 10 μM FeSO₄. For iron-depleted conditions, iron was omitted. The blood agar was –Fe-AMM containing 1.5 mM FeSO₄ and the nitrogen source, and 10 μM FeSO₄. For iron-depleted conditions, iron was omitted. The blood agar was –Fe-AMM containing 1.5 mM FeSO₄ and the nitrogen source, and 10 μM FeSO₄. For iron-depleted conditions, iron was omitted. The blood agar was –Fe-AMM containing 1.5 mM FeSO₄ and the nitrogen source, and 10 μM FeSO₄. 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Table I. A. fumigatus Strains Used in This Study

| Strain          | Genotype        | Reference |
|-----------------|-----------------|-----------|
| CEA10           | Wild type       | 22        |
| CEA17           | pyrG⁻           | 22        |
| ΔsidA-CEA17     | CEA17, ΔsidA:pyrG | This work |
| sidA⁻-CEA17     | ΔsidA-CEA17, (p):sidA | This work |
| ΔsidA/ΔftrA-CEA17| ΔsidA-CEA17, ΔftrA:hph | This work |
| ΔftrA-CEA17     | ΔsidA/ΔftrA-CEA17, (p)::sidA | This work |
| ATCC 46645      | Wild type       | American Type Culture Collection |
| ΔsidA           | ATCC 46645, ΔsidA:hph | This work |
| ΔftrA           | ATCC 46645, ΔftrA:hph | This work |
| SidA³           | ΔsidA, ΔsidA::sidA* | This work |

* indicates a silently mutated version of sidA (see Materials and Methods).
³ indicates a reconstituted genomic locus.
⁻ indicates the presence of an ectopically integrated, complementing allele.
site allows reconstituted strains to be differentiated from wild-type contaminants. A representative transformant, SidA⁺, was selected for further analysis.

To enable inactivation of ftrA, a 5.0-kb fragment of genomic DNA containing *A. fumigatus* ftrA was amplified using primers 5'−GTTGGGATTGCTAGTGCAG and 5'−AGAATTGATAT-CAACACCTTTCCCATATAAC. The resulting plasmid was sequenced and designated pFTRA. An internal 1.7-kb NheI-HindIII fragment (−395 to + 2082, stop codon at +1322) was replaced with a 3.2-kb NheI-HindIII fragment from pAN7-1 carrying *hyg*. A gel-purified 6.5-kb EcoRV fragment was used to transform *A. fumigatus* sidA−CEA17, sidA−CEA17, and ATCC 46645. Hygromycin-resistant *ftrA*− transformants were selected, and a representative strain from each background, ΔsidA/ΔftrA-CEA17, ΔftrA-CEA17, and ΔftrA, respectively, were selected and used for further analysis.

**Virulence Assays.** CD1 male mice (18–22 g; Harlan UK Ltd.) were housed in groups of eight in individually vented cages. Animals were allowed free access to food and water containing 1 g/liter tetracycline hydrochloride and 64 mg/l ciprofloxacin as prophylaxis against bacterial infection. Mice were immunosuppressed with i.p. cyclophosphamide (150 mg/kg) on days −3, −1, +2, and every subsequent third day throughout each experiment. A single dose of hydrocortisone acetate (112.5 mg/kg) was administered s.c. on day −1. Conidiospores were harvested in sterile saline from *Aspergillus* complete medium slants (containing 5 mM ammonium (−tartarate, 1.5 mM FeSO₄, and 200 mM NaOHPO₄) incubated for 5 d at 37°C. The resultant suspension was filtered through Miracloth (Calbiochem), washed twice with sterile saline, counted using a hemocytometer, and resuspended at 10⁹ spores/ml. Mice were anesthetized with isoflurane and infected by intranasal instillation of 4 × 10⁶ conidiospores in 40 μl of saline. Mice were weighed at 24-h intervals from day 0. Visual inspections were made twice daily. Mice were killed when predetermined end points, e.g., 20% weight-loss, moribund state, or failure to respond to gentle stimuli, were reached. Survival curves were compared using Kaplan-Meier log rank analysis. Immediately after sacrifice, lungs were removed and fixed in 4% formaldehyde (Sigma-Aldrich). Lungs were embedded in paraffin before sectioning and staining with hematoxylin and eosin and light green Grocott’s methenamine silver. Murine infections were performed under UK Home Office Project Licence PPL/70/5361 in dedicated facilities at Imperial College London.

**Results and Discussion**

**SidA Is Essential for Siderophore Biosynthesis.** In a first step to studying the role of the siderophore system in iron homeostasis and virulence in *A. fumigatus*, we analyzed production of the hydroxamate-type siderophores TAF and DFFC in two *A. fumigatus* isolates, ATCC 46645 and CEA10, by reversed phase HPLC (21). During iron starvation, both strains secrete TAF and accumulate intracellular DFFC (Fig. 1 A). In iron-replete conditions, production of both siderophores is low. *A. fumigatus* siderophore production therefore resembles that of *A. nidulans* (21). Recently, we have shown that 1-ornithine-NO₃⁻-monooxygenase, which catalyzes the first committed step of hydroxamate-type siderophore biosynthesis, is essential for growth of *A. nidulans* (18). A search in the genome sequence of *A. fumigatus* (http://www.tigr.org) revealed one putative 1-ornithine-NO₃⁻-monooxygenase encoding gene, termed sidA. Comparison of the genomic and cDNA sequences revealed the presence of one intron in sidA (sequence data available from GenBank/EMBL/DDJ under accession no. AY586511). The predicted amino acid sequence of SidA contains 501 amino acids, possesses all signatures typical for hydroxylases involved in siderophore biosynthesis, and has 78% identity to *A. nidulans* SidA. Northern analysis shows that *A. fumigatus* sidA expression is up-regulated by iron starvation (Fig. 1 B).

To analyze the function of SidA, we constructed gene deletions in both *A. fumigatus* backgrounds. HPLC analysis showed that the resulting sidA-deficient strains lose the
Figure 2. (A) Growth phenotypes of *A. fumigatus* wild-type and mutant strains. SidA and FtrA are components of two independent iron uptake mechanisms. Aliquots of 10⁴ conidia of the respective strain were point inoculated on AMM plates containing the respective iron source and incubated for 48 h at 37°C. Blood agar was AMM containing 5% (vol/vol) sheep blood. (B) Quantitative analysis of radial growth of *A. fumigatus* wild-type (CEA10 [1], ATCC 46645 [6]) and mutant strains (ΔsidA-CEA17 [2], ΔsidA-c-CEA17 [3], ΔftrA-CEA17 [4], ΔsidA/ΔftrA-CEA17 [5], ΔsidA [7], ΔsidA⁰ [8], ΔftrA [9]) was performed following growth for 48 h at 37°C. *A. fumigatus* CEA10 radial growth on 10 μM FeSO₄ was normalized to 100%. (C) Quantitative analysis of conidiation of *A. fumigatus* wild-type (CEA10 [1], ATCC 46645 [6]) and mutant strains (ΔsidA-CEA17 [2], ΔsidA-c-CEA17 [3], ΔftrA-CEA17 [4], ΔsidA/ΔftrA-CEA17 [5], ΔsidA [7], ΔsidA⁰ [8], ΔftrA [9]) was performed following growth for 120 h at 37°C. *A. fumigatus* CEA10 conidiation rate on 10 μM FeSO₄ was normalized to 100%.
ability to produce both TAFC and DFFC (Fig. 1 C), demonstrating that sidA is required for A. fumigatus siderophore biosynthesis and is highly likely to encode l-ornithine-N\(^{\delta}\)-monooxygenase. In contrast to a siderophore-negative A. nidulans strain, which is almost nonviable without siderophore supplementation (18), sidA deficiency in A. fumigatus decreases growth rates moderately during iron starvation and only slightly in iron replete conditions (Fig. 2; unpublished data). However, a complete lack of growth was observed on blood agar plates (Fig. 2). This was rescued by addition of FC to the medium. In iron-depleted and replete conditions, the sidA-deficient strains had only 1% of wild-type conidiospore production; this increased to \(\approx 50\%\) by supplementation with 1.5 mM iron. Supplementation with FC or complementation of sidA in both backgrounds rescued all observed phenotypes (Fig. 2; unpublished data), e.g., defects in conidiation and growth on blood agar plates, demonstrating that the sidA\(^{-}\) phenotypes are a direct result of loss of sidA.

A. fumigatus can acquire iron by reductive assimilation and ferrous uptake but lacks specific mechanisms for uptake of host iron compounds. The ability of A. fumigatus sidA\(^{-}\) strains to grow on iron-limited media indicate that, in contrast to A. nidulans, this fungus possesses at least one additional iron assimilation system. Further inspection of the A. fumigatus genome sequence revealed the presence of several putative metalloredoxase-encoding genes, one putative ferroxidase fetC, and one potential high affinity iron permease-encoding gene ftrA. fetC and ftrA are divergently transcribed from a 1.3-kb intergenic region and comparison of the genomic and cDNA sequences revealed the presence of five introns in fetC and three introns in ftrA (sequence data available from GenBank/EMBL/DDBJ under accession no. AY586512). The deduced amino acid sequences of FetC and FtrA are 52 and 55% identical, respectively, to the C. albicans ferroxidase Fet3 and iron permease Ftr1 (25). Northern blot analysis shows that expression of both genes is up-regulated by iron starvation (Fig. 1 B). Furthermore, A. fumigatus sidA\(^{-}\) mutants exhibit increased sensitivity to two inhibitors of the reductive iron uptake system, bathophenanthroline disulfonate, and copper depletions (unpublished data; 26). Together, these data suggest that unlike A. nidulans, A. fumigatus has the capacity for reductive iron assimilation.

To analyze the potential role of FtrA in iron uptake by A. fumigatus, the ftrA gene was deleted in both A. fumigatus backgrounds. These ftrA-deficient strains have essentially wild-type growth and conidiation phenotypes (Fig. 2). However, they display an eightfold increase in TACF production after 12 h of growth in iron-depleted conditions, demonstrating that lack of FtrA brings forward the onset of siderophore production, possibly to compensate for the lack of reductive iron assimilation.

We also constructed a \(\Delta sidA/\Delta ftrA\) double mutant in A. fumigatus CEA17. On most media, this strain is unable to grow unless supplemented with ferricrocin (Fig. 2), demonstrating that ftrA encodes an essential component of a siderophore-independent iron uptake system. Very slow growth occurs in the presence of high levels of ferrous, but not ferric, iron (Fig. 2), suggesting the presence of a specific SidA/FtrA-independent ferrous iron uptake system. In addition, the \(\Delta sidA/\Delta ftrA\) double mutant failed to grow on blood agar plates (Fig. 2) or on media containing 10 \(\mu\)M of hemoglobin, hemin, holotransferrin, or ferritin as the sole iron source (unpublished data). Thus, as an opportunistic rather than committed pathogen, A. fumigatus lacks specific systems for the uptake of host iron compounds.

**Siderophore Biosynthesis But Not Reductive Iron Assimilation Is Essential for Virulence of A. fumigatus.** To test the requirement for sidA and ftrA in a murine model of invasive aspergillosis, we compared the virulence of wild-type parental A. fumigatus ATCC 46645 and the otherwise isogenic \(\Delta sidA\) and \(\Delta ftrA\) strains. Groups of 15 neutropenic CD1 mice were intranasally inoculated with equivalent doses (\(2 \times 10^5\)) of conidiospores from each strain. The \(\Delta ftrA\) mutant was as virulent as the wild-type strain, demonstrating that reductive iron uptake has little or no role to play in A. fumigatus virulence (Fig. 3). All mice infected with A. fumigatus \(\Delta ftrA\) and ATCC 46645 displayed significant weight loss within 24 h of infection, and weight dropped further throughout the course of the experiment. In vivo, germination of both strains was detectable 40 h postinfection (not depicted) and mice infected with either strain exhibited >90% mortality within 6 d of infection (Fig. 3). A. fumigatus was cultured from the lungs of all killed animals. Mock infection with saline resulted in zero mortality (not depicted). In stark contrast, the \(\Delta sidA\) mutant is completely attenuated (Fig. 3). Animals infected with A. fumigatus \(\Delta sidA\) demonstrated modest weight loss within 24 h of infection. This was followed by steady weight gain, despite continued immunosuppression, for the duration of the experiment. All mice survived to at least 15 d postinfection (\(P < 0.001\); Kaplan Meier Log Rank Analysis). Reconstitution of sidA resulted in restoration of virulence to wild-type levels (Fig. 3), demonstrating that sidA, and thus siderophore biosynthesis, is absolutely required for A. fumigatus virulence.

To investigate differences in the fate of A. fumigatus sidA-null (\(\Delta sidA\)) and reconstituted (sidA\(^R\)) conidiospores in vivo and assess variation in host immune responses, mice were killed 20, 40, and 60 h postinfection with \(2 \times 10^5\) A. fumigatus conidiospores, from each strain. The determined mortality (closed circles) is completely attenuated for virulence (Fig. 3). All mice infected with A. fumigatus \(\Delta sidA\) and ATCC 46645 displayed significant weight loss within 24 h of infection, and weight dropped further throughout the course of the experiment. In vivo, germination of both strains was detectable 40 h postinfection (not depicted) and mice infected with either strain exhibited >90% mortality within 6 d of infection (Fig. 3). A. fumigatus was cultured from the lungs of all killed animals. Mock infection with saline resulted in zero mortality (not depicted). In stark contrast, the \(\Delta sidA\) mutant is completely attenuated (Fig. 3). Animals infected with A. fumigatus \(\Delta sidA\) demonstrated modest weight loss within 24 h of infection. This was followed by steady weight gain, despite continued immunosuppression, for the duration of the experiment. All mice survived to at least 15 d postinfection (\(P < 0.001\); Kaplan Meier Log Rank Analysis). Reconstitution of sidA resulted in restoration of virulence to wild-type levels (Fig. 3), demonstrating that sidA, and thus siderophore biosynthesis, is absolutely required for A. fumigatus virulence.
conidia. Lungs were surgically removed and prepared for histology (Fig. 4). Conidia of both strains remain largely ungerminated in the bronchioles 20 h postinfection, and similar levels of cellular recruitment to sites of conidial deposition can be seen. Germination and hyphal extension of the reconstituted sidA<sup>R</sup> conidia are clearly visible 40 h postinfection. Some evidence of epithelial penetration by hyphae, accompanied by increased inflammatory infiltration, is also apparent. At 60 h postinfection, considerable mycelial growth of <i>A. fumigatus</i> sidA<sup>R</sup> can be seen in the lung, characterized by extensive penetration of surrounding tissues, effectively destroying any recognizable lung structure at the foci of infection, and heavy recruitment of inflammatory cells (Fig. 4). In complete contrast, no germination of ΔsidA conidia was observed in vivo (Fig. 4). Although conidia are still present in bronchioles 60 h postinfection, only very limited cellular recruitment to foci of infection was seen (Fig. 4). These data suggest that the process of germination plays a role in inducing the host’s inflammatory response to infection.

Our data demonstrate that <i>A. fumigatus</i> principally employs a siderophore-based iron acquisition system in vivo, in contrast to the reductive iron assimilation system required in the mammalian host by <i>C. albicans</i> (11). Indeed the <i>A. fumigatus</i> reductive iron assimilation system is redundant in vivo. In addition to <i>A. fumigatus</i>, many pathogenic fungi produce hydroxamate-type siderophores (27). Since mammals lack SidA, and the hydroxamate-type siderophore system in general, it represents a possible target for the development of antifungal therapies against siderophore-producing fungi.

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No author has a direct competing interest in the work presented.

A patent application has been filed on aspects of this work, and some authors potentially have patent rights.

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