Biosynthesis of an extracellular siderophore is essential for maintenance of mutualistic endophyte-grass symbioses

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Abstract

We have identified a novel siderophore from the mutualistic grass symbiont *Epichloë festucae*. Targeted gene replacement of a non-ribosomal peptide synthetase (termed *sidF)* from *E. festucae* eliminated biosynthesis of this extracellular siderophore. Structural characterisation, based on extensive mass spectrometry showed similarities to fusarinine-type siderophores. Plants inoculated with strains carrying a targeted deletion in *sidF* have a dramatic phenotype. Siderophore loss changed a mutualistic symbiont into an antagonistic fungus, causing de-regulation and proliferation of fungal hyphae, plant stunting and sometimes even tiller death. We hypothesise that loss of the biosynthesis of the siderophore disrupts iron homeostasis within the whole symbiotum, which is a critical factor for maintenance of mutualistic endophyte-grass symbioses.

Keywords: siderophore, iron homeostasis, NRPS

Introduction

Iron is an essential trace element required for nearly all living organisms. Due to its insolubility and toxicity in aerobic environments, iron is not freely available in living tissues. Under iron starvation, most micro-organisms synthesise siderophores - low molecular weight, iron-chelating agents that solubilise iron (III) and control intracellular iron levels (Haas 2003; Renshaw *et al.* 2002). Some siderophores also function as virulence factors (Oide *et al.* 2006; Ratledge & Dover 2000).

Endophytic fungi of the genus *Epichloë* (Ascomycota, Clavicipitaceae) and related asexual *Neotyphodium* species form mutualistic symbioses with temperate grasses (Poaceae). The symbiont's growth is confined to the intercellular spaces of leaf sheaths and blades. This symbiosis can be mutually beneficial and numerous biotic and abiotic advantages are conferred upon the host by the fungus (Schardl *et al.* 2004).

Most fungi produce hydroxamate-type siderophores (van der Helm & Winkelmann 1994), which are typically composed of three hydroxamate groups linked by peptide or ester bonds to form an octahedral complex. Linkage of these groups is catalysed by non-ribosomal peptide synthetases (NRPS) which may also add additional amino acids (Plattner & Diekmann 1994). These multifunctional enzymes are modular in structure whereby a typical module consists of a substrate adenylation domain, a peptidyl carrier domain, and a condensation domain that catalyses peptide bond formation (Finking & Marahiel 2004). A few NRPS genes from filamentous fungi have been shown to encode NRPSs responsible for the synthesis of siderophores (Eisendle *et al*. 2003; Yuan *et al*. 2001; Oide *et al*. 2006; Tobiasen *et al.* 2006). Most notably, the NRPS encoded by *NPS6* from *Cochliobolus heterostrophus*, *Fusarium graminearum* and *Alternaria brassicicola* (Oide *et al*. 2006) is responsible for biosynthesis of extracellular siderophores of the coprogen/fusarinine-type which was demonstrated to have a role in virulence to plants.

Methods

Molecular biology

An *Epichloë festucae* (FL1) fosmid library was constructed (Epicentre Biotechnologies). A gene replacement construct was created using Multisite Gateway Three-fragment Vector Construction Kit (Invitrogen). Entry clones were combined to produce a destination vector containing inserts of approximately 3 kb of 5' NRPS coding sequence, followed by a hygromycin resistant gene and approximately 3 kb of consecutive NRPS sequence. Protoplasts were prepared as described by (Tanaka *et al.* 2005and references within) and transformed using methodology of Vollmer & Yanofsky (1986) and modifications by Itoh *et al.* (1994).

Culture conditions

Media for iron growth studies and chemical analyses were modified from (Mantle & Nisbet 1976), with yeast extract replaced with 0.6 µM thymine. Iron was omitted for iron-depleted media or supplemented with $FeSO_4$ or $FeCl_3$ as indicated.

LCMSMS

Samples were extracted from both culture supernatant and freeze-dried mycelia from iron-depleted and iron-supplemented liquid cultures, as described above, using the SPE method (McCormack *et al.* 2003). Analytes were eluted through a C18 Luna column (Phenomenex Torrence, CA, USA) (150 x 2 mm, 5µm) at a flow rate of 200 µl min^{-1} using a Thermo Finnigan Surveyor HPLC system with a solvent gradient (solvent A: H_2O 0.1% formic acid; B: MeCN 0.1% formic acid), starting with 3% B, 97% A for 5 minutes and then increasing to 23% B over 15 minutes followed by a column wash at 95% B. UV spectra were obtained using a Thermo surveyor PDA detector (200-600 nm) and monitored at

Figure 1 Modular organisation of *E. festucae* SidF, *Aspergillus nidulans* SidC and *Ustilago maydis* Sid2. A: adenylation domain, P: peptidyl carrier protein, C: condensation domain. C*: non-functional condensation domain.

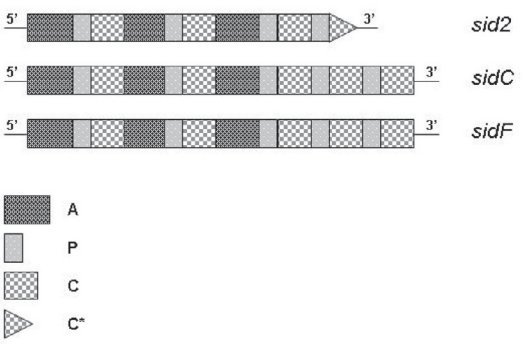
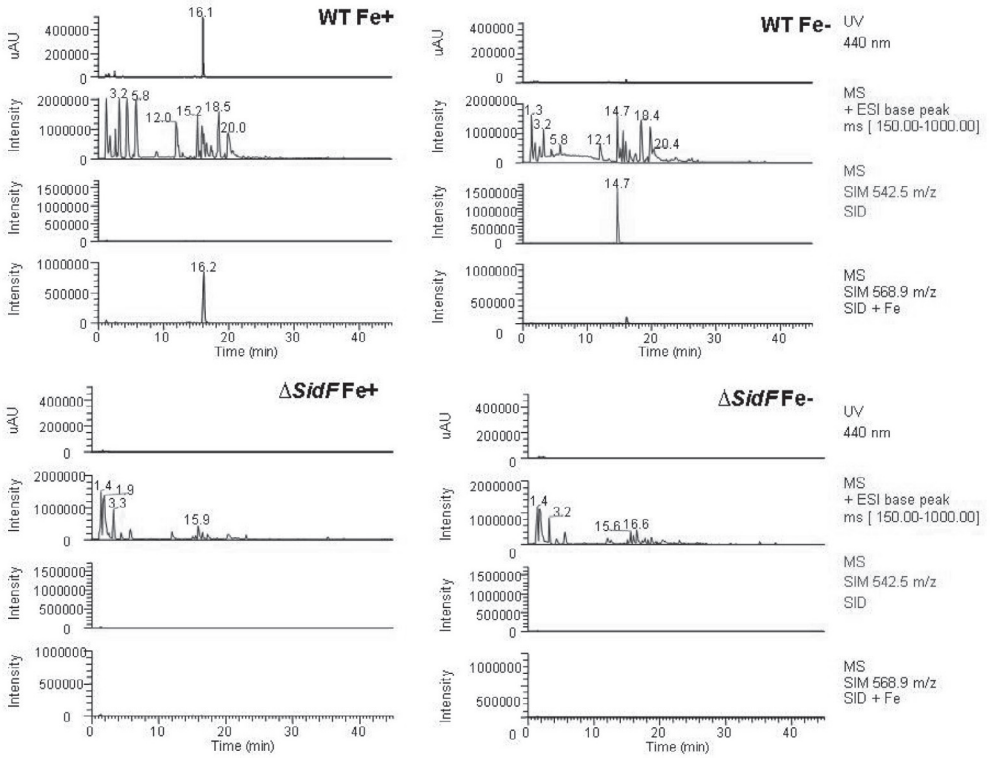


Figure 2 Comparison of wild-type (WT) and mutant (Δ*sidF*) chromatograms from supernatants extracted from iron depleted (Fe-) or iron supplemented (Fe+) media. Peak at 14.7 represents the iron-free form of the siderophore and the peaks at 16.1 or 16.2 represents the iron-bound form.



440 nm, which is typical for siderophore-Fe^{3+} complexes.

Mass spectra were determined with a linear ion trap mass spectrometer (Thermo LTQ) using ESI in +ve mode. The spray voltage was 5.0 kV and the capillary temperature 275°C. The flow rates of sheath gas, auxiliary gas, and sweep gas were set to 20, 5, and 10 (arbitrary units), respectively. Other parameters were optimised automatically by infusing ferrichrome in H2O: MeCN:HCOOH (75:25:0.1, v/v/v) at a flow rate of 200 µl min^{-1}.

Results

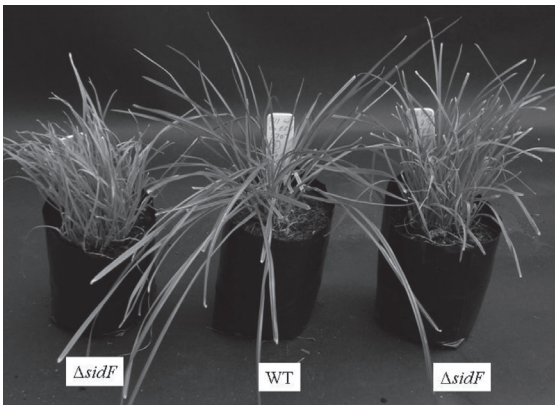
A degenerate PCR strategy was undertaken to generate a library of endophyte NRPS sequences (Johnson *et al*. 2007). Characterisation of one of these NRPSs (termed *sidF*) is presented here. The promoter and entire open reading frame of *sidF* was obtained from an *E. festucae* fosmid library. A BlastX analysis of the deduced amino acid sequence revealed high identities to fungal NRPS genes involved in siderophore biosynthesis. The domain structure of *sidF* predicts the presence of three adenylation domains (Fig. 1).

To investigate whether *sidF* encodes a NRPS gene involved in siderophore biosynthesis, a gene replacement was performed by homologous recombination. Replacement mutants were selected and phenotypic growth characteristics were examined *in vitro*. On iron-depleted media supplemented with 100 µM of bathophenanthrolinedisulfonic acid (an iron chelator), the wild-type strain's growth was not affected, but all mutant strains were unable to grow; a phenotype expected if no extracellular siderophores are synthesised. Addition of either 80 µM Fe2+ or Fe3+ could overcome this growth deficiency.

To determine the metabolic end-product of *sidF*, chemical analysis was carried out on both wild-type and knock-out strains. Both culture supernatant and mycelial extracts were analysed by LCMSMS coupled to PDA. Comparison of the wild-type and mutant chromatograms from the supernatant extractions clearly showed the absence of an extracellular siderophore in the mutant strains, indicating that *sidF* encodes an extracellular siderophore (Fig. 2). Extensive mass spectral studies, using $LCMS^n$ and ICRTFMS (data not shown) indicated that this compound has three identical units consisting of a hydroxamate attached through the acyl group to anhydromevalonic acid and three additional amino acids. From our structural information, we believe the endophyte siderophore to be a novel member of the fusarinine family.

To find out if loss of *sidF* has an effect on the symbiotic relationship of *E. festucae* with perennial ryegrass, *sidF* mutant strains were inoculated into ryegrass seedling lines. Plants infected with siderophore mutant strains gave a surprisingly dramatic phenotype (Fig. 3). Microscopic examination of aniline-blue stained leaf sheaths from the mutant revealed on-going tip growth and excessive hyphal branching in the expanding leaf zone resulting in an increase in fungal biomass and loss of synchronisation of endophyte growth with that of its host plant. In addition, plant stunting and occasionally tiller death occurred. Transmission electron microscopy revealed an altered hyphal ultrastructure, including irregular hyphal shape and uncharacteristic vacuolation.

Figure 3 Comparison of wild-type (WT) and individual siderophore minus mutants (Δ*sidF*) associated with perennial ryegrass.



Discussion

No siderophores or genes involved in their biosynthesis have been reported previously from grass endophytes. We identified and characterised a fungal NRPS gene, *sidF,* from *E. festucae* and show that it is required for the biosynthesis of a novel hydroxamate siderophore, most likely of the fusarinine-type. Chemical analysis, combined with the inability of the *sidF* mutant to grow on iron-depleted media, indicates the presence of just one extracellular siderophore. Most significantly, associations of the siderophore mutant strains of the symbiotic endophyte with perennial ryegrass are detrimental and disrupt the symbiosis. In contrast, loss of extracellular siderophores through deletion of the NRPS gene *NPS6* from fungal plant pathogens resulted in reduced virulence (Oide *et al*. 2006). We hypothesise that lack of the endophyte extracellular siderophore alters iron homeostasis of the symbiotum, perturbing the natural interaction and changing it from mutually beneficial to antagonistic.

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