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Contrasting roles of fungal siderophores in maintaining iron homeostasis in *Epichloë festucae*

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ABSTRACT

The symbiosis between *Epichloë festucae* and its host perennial ryegrass (*Lolium perenne*) is a model system for mutualistic interactions in which the fungal endophyte grows between plant shoot cells and acquires host nutrients to survive. *E. festucae* synthesises the siderophore epichloënin A (EA) via SidN, a non-ribosomal peptide synthetase (NRPS). EA is involved in the acquisition of iron, an essential micronutrient, as part of the process of maintaining a stable symbiotic interaction. Here, we mutated a different NRPS gene *sidC* and showed that it is required for production of a second siderophore ferricrocin (FC). Furthermore mutations in *sidA,* encoding an L-ornithine N^5-monooxygenase, abolished both EA and FC production. Axenic growth phenotypes of the siderophore mutants were altered relative to wild-type (WT) providing insights into the roles of *E. festucae* siderophores in iron trafficking and consequently in growth and morphogenesis. During iron-limitation, EA is the predominant siderophore and in addition to its role in iron acquisition it appears to play roles in intracellular iron sequestration and oxidative stress tolerance. FC in contrast is exclusively located intracellularly and is the dominant siderophore under conditions of iron sufficiency when it is likely to have roles in iron storage and iron transport. Intriguingly, EA acts to promote but may also moderate *E. festucae* growth (depending on the amount of available iron). We therefore hypothesise that coordinated cellular iron sequestration through FC and EA may be one of the mechanisms that *E. festucae* employs to manage and restrain its growth in response to iron fluxes and ultimately persist as a controlled symbiont.

1. Introduction

Iron is an essential, yet potentially cytotoxic micronutrient crucial for metabolic viability in all eukaryotes (Kaplan and Kaplan, 2009). Fungi employ multiple specialised cellular systems to acquire iron from iron-limiting environments including high affinity iron-chelating molecules (siderophores) and/or membrane-bound reductive iron assimilation (RIA) systems (Haas et al., 2008; Johnson, 2008). Free iron can participate as redox catalysts in cell-damaging reactive oxygen-generating reactions (Halliwell and Gutteridge, 1992). Fungi lacking dedicated iron excretion systems sequester and store surplus iron intracellularly either as ferric-chelates that are contained in vacuoles or as Fe^{3+}-siderophores (Raguzzi et al., 1988; Li et al., 2001; Haas et al., 2008). Thus, iron acquisition, utilisation and storage processes are strictly regulated to avoid detrimental stresses associated with iron concentration extremes.

Siderophores are small chelator molecules with extremely high affinity for ferric (Fe^{3+}) iron (Neilands, 1993). Secreted (extracellular) siderophores are deployed for iron uptake while intracellular siderophores contribute toward iron storage, sequestration and as iron transporters. Siderophores have been shown to have roles in resistance to oxidative stress and host virulence (Haas et al., 2008; Johnson, 2008). Most fungi produce hydroxamate-containing siderophores and the ferrichrome-type siderophores typically have three L-ornithine-derived hydroxamate units (N^5-acyl-N^5-hydroxyornithine) that coordinate a single ferric ion and which are assembled together with conventional amino acids by non-ribosomal peptide synthetase (NRPS) enzymes in the latter steps of siderophore biosynthesis (Van Der Helm and Winkelmann, 1994; Haas et al., 2008).

In the first step of hydroxamate synthesis (Fig. 1A), L-ornithine is converted by L-ornithine N^5-monooxygenase (OMO) to N^5-hydroxy-L-ornithine, the common precursor of all hydroxamate siderophores. The hydroxamate amino acid is then formed by a N^5-transacylase enzyme joining an acyl group, most commonly acetate or Δ^2-anhydromevalonate to the precursor molecule, with the acyl group thus specifying the hydroxamate class (Jalal and van der Helm, 1991). NRPSs are large modular enzymes that can produce a diverse range of metabolites using a ribosome-independent thiotemplate mechanism

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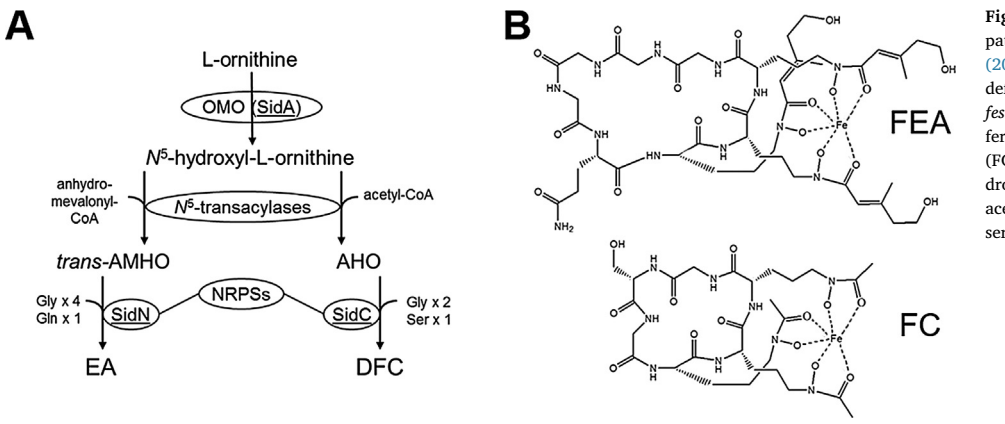

Fig. 1. (A) A model of the siderophore biosynthesis pathways in *E. festucae* based on Johnson et al. (2013b), producing EA and desferricrocin (DFC, i.e., deferrated forms). (B) Schematic representation of *E. festucae* siderophores EA and FC, shown here bound to ferric iron as ferriepichloënin A (FEA) and ferricrocin (FC), respectively. Abbreviations: AMHO, N^5-anhydromevalonyl-N^5-hydroxyl-L-ornithine; AHO, N^5-acetyl-N^5-hydroxyl-L-ornithine; Gly, glycine; Ser, serine; Gln, glutamine.

from at least three functional domains (Marahiel et al., 1997; Marahiel, 2009). The adenylation (A) domain specifically activates the appropriate amino acid substrate which is then covalently attached to the thiol group of a cofactor, 4-phosphopantetheine via the thiolation (T) domain/peptidyl carrier protein (PCP) domain while the condensation (C) domain forms the peptide bond with subsequent transfer of the growing peptide to the next module (Finking and Marahiel, 2004). The adenylation domain determines substrate specificity via key pocket-lining, substrate-binding amino acid residues (Stachelhaus et al., 1999; Schwecke et al., 2006; Bushley et al., 2008; Lee et al., 2010).

Members of the fungal genus *Epichloë* (Ascomycota: Clavicipitaceae) are non-invasive, exclusively foliar intercellular fungal endophytes of graminaceous plants of the subfamily *Pooideae* (family: Poaceae) (Hinton and Bacon, 1985; White et al., 1993; Leuchtmann et al., 2014). These symbiotic associations form a continuum of interactions from mutualistic to antagonistic, biased toward mutualistic interactions and are generally spread to their new hosts via vertical transmission in seed tissues (Schardl, 1996; Majewska-Sawka and Nakashima, 2004; Schardl et al., 2004). The hyphae grow attached to, and in synchrony with, their neighbouring host cells (Christensen et al., 1997, 2008) and nutrients are assimilated from the host apoplasm by unknown mechanisms. These fungi are well-known for their capacity to produce an array of strain-specific metabolites, bestowing resistance to a range of biotic and abiotic stresses leading to increased pasture persistence for mutual benefit (Bush et al., 1997; Blankenship et al., 2001; Panaccione et al., 2001; Spiering et al., 2005; Tanaka et al., 2005; Young et al., 2006), and are agronomically valued (Johnson et al., 2013a).

We previously characterised the siderophore synthetase (SidN) that produces a structurally unique secreted ferrichrome-type siderophore, designated epichloënin A (EA, Fig. 1B) and a minor variant epichloënin B (Koulman et al., 2012; Johnson et al., 2013b). EA has roles in iron acquisition and in maintaining mutualism between *E. festucae* and perennial ryegrass (*Lolium perenne* L. 1953) (Schardl, 2001; Johnson et al., 2013b). This was the first account that loss of a siderophore could cause an endophytic fungus to antagonise its host, inducing morphological changes of the grass-endophyte symbiotum and premature senescence of the grass host (Johnson et al., 2013b). In the course of these investigations, a second major siderophore, ferricrocin (FC, Fig. 1) was identified by LCMS in cultures of *Epichloë festucae* var *lolii* ((Fr.) Tul & C. Tul., 1865) (L. Johnson, A. Koulman, unpublished, Fig. S1), although the evidence establishing the NRPS gene responsible for its biosynthesis was incomplete. FC functions mostly as an intracellular siderophore in filamentous fungi, and FC mutants often display sensitivity to oxidative stress during iron starved conditions (Eisendle et al., 2003; Schrettl et al., 2007). This study seeks to dissect siderophore biosynthesis and the functionality of the siderophores produced by *E. festucae*. Mutants were generated that were deficient in siderophore biosynthesis (SB), and their phenotypes characterised with respect to iron supply, response to oxidative stress and siderophore production.

2. Materials and methods

2.1. Fungal strains, media and axenic growth conditions

Epichloë festucae (Leuchtmann et al., 2014) strain Fl1(*ex-cultivar SR3000*) used in this study was isolated from *L. perenne*, whereas strain Fl1 (Johnson et al., 2013b) was isolated from meadow fescue (*Festuca pratensis*) using the grow-out method of Clark et al. (1983). A list of fungal strains used in this study is presented in Table S1. Axenic culturing of fungi was performed at 22 °C in an 8 h light, 16 h dark cycle from 3 to 30 days as indicated. Cultures were maintained on 2.4% potato dextrose agar or broth (PDA or PDB, Difco Laboratories, France). Defined medium (DM) used as a nutrient base for fungal growth studies and chemical analyses was prepared as described previously Johnson et al. (2013b). For iron growth studies DM medium was supplemented with sterile additives to either induce iron starvation (100 μM BPS; bathophenanthroline disulfonic acid; Sigma) or to provide iron (in the form of $FeCl_3$) to low (1–5 μM), adequate (20 μM), sufficient (50 μM) or excess (250 μM) concentrations for the respective experiments. Additionally, 1.7–2.0 mM H_2O_2 was added to DM0 to induce oxidative stress to *E. festucae* in tests performed as described in Johnson et al. (2013b). Growth assays were performed at least three times, with colonies grown for 10–14 days at 23 °C before colony diameter measurements were taken. Representative colonies from one of these assays of the study are shown in the results.

Starter cultures of the *E. festucae* strains were prepared from plugs of 10 day-old PDA-grown hyphae (36 × 2.5 mm) which were harvested from the colony leading edge, macerated using a micro-pestle and added to 50 mL PDB. To prevent formation of large mycelial clumps, mycelia were collected after three days growth (23 °C, 100 rpm) by centrifugation (2500*g*, 5 min), re-macerated and inoculated into fresh 50 mL PDB and re-incubated (23 °C, 2 days, 100 rpm). The mycelia were collected by centrifugation (2500*g*, 5 min) and washed twice in 50 mL DM0 (30 min stand followed by 2500*g*, 10 min), suspended in DM0 at 4 volumes per gram of fresh weight pellet and used as indicated in each experiment.

2.2. Nucleic acid preparations and standard molecular techniques

Plasmid or fosmid DNA maintained in *Escherichia coli* TOP10 strain (Invitrogen) were prepared using standard methods (Qiagen & Epicentre Biotechnologies). Crude genomic DNA was prepared as templates to identify potential mutants by PCR as previously described (Johnson et al., 2013b). High quality fungal genomic DNA (gDNA) were isolated using the method of Yoder (1988) and subjected to Southern blot analyses (DIG, Roche). Standard PCR reactions were performed in 20 μL using Platinum Taq DNA polymerase (Invitrogen) mix with 1 mM $MgCl_2$ and 0.25 mM of each primer. PCR products used for construct creation (deletion flanks and complementing DNAs) in *E. coli* and for *E.*