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Research paper

The rhizoferrin biosynthetic gene in the fungal pathogen *Rhizopus delemar* is a novel member of the NIS gene family

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ABSTRACT

Iron is essential for growth and in low iron environments such as serum many bacteria and fungi secrete ferric iron-chelating molecules called siderophores. All fungi produce hydroxamate siderophores with the exception of Mucorales fungi, which secrete rhizoferrin, a polycarboxylate siderophore. Here we investigated the biosynthesis of rhizoferrin by the opportunistic human pathogen, *Rhizopus delemar*. We searched the genome of *R. delemar* 99–880 for a homologue of the bacterial NRPS-independent siderophore (NIS) protein, SfnaD, that is involved in biosynthesis of staphyloferrin A in *Staphylococcus aureus*. A protein was identified in *R. delemar* with 22% identity and 37% similarity with SfnaD, containing an N-terminal IucA/IucC family domain, and a C-terminal conserved ferric iron reductase FhuF-like transporter domain. Expression of the putative fungal rhizoferrin synthetase (*rfs*) gene was repressed by iron. The *rfs* gene was cloned and expressed in *E.coli* and siderophore biosynthesis from citrate and diaminobutane was confirmed using high resolution LC–MS. Substrate specificity was investigated showing that Rfs produced AMP when oxaloacetic acid, tricarballylic acid, ornithine, hydroxylamine, diaminopentane and diaminopropane were employed as substrates. Based on the production of AMP and the presence of a mono-substituted rhizoferrin, we suggest that Rfs is a member of the superfamily of adenylating enzymes. We used site-directed mutagenesis to mutate selected conserved residues predicted to be in the Rfs active site. These studies revealed that H484 is essential for Rfs activity and L544 may play a role in amine recognition by the enzyme. This study on Rfs is the first characterization of a fungal NIS enzyme. Future work will determine if rhizoferrin biosynthesis is required for virulence in Mucorales fungi.

1. Introduction

Mucormycosis is a potentially life-threatening infection in immunocompromised individuals caused by Mucorales fungi. The most commonly-isolated clinical specimens belong to the genera *Rhizopus, Mucor, Cunninghamella* and *Lichtheimia* (Alvarez et al., 2009; Park et al., 2011) though *Rhizopus* species account for up to 70% of mucormycosis infections (Roden et al., 2005). Some species within *Rhizomucor, Saksenaea* and *Apophysomyces* are also pathogenic but are less commonly isolated (Roden et al., 2005). As Mucorales fungi are opportunistic pathogens, they affect immunocompromised individuals such as those with hematological malignancies, particularly acute myeloid leukemia (Pagano et al., 1997), as well as hematopoietic stem cell transplant recipients (HSCT) (Sun and Singh, 2008), patients with uncontrolled diabetes (Roden et al., 2005) or those with elevated serum iron levels (Boelaert et al., 1994; Spellberg et al., 2005). Patients with diabetic ketoacidosis (DKA) are particularly susceptible to mucormycosis and the ability to colonize DKA patients is unique to Mucorales fungi; *Aspergillus* and *Candida* infections are uncommon in this patient group (Liu et al., 2010). Liposomal amphotericin B and posaconazole are the main antifungal agents used in chemotherapy (Cornely et al., 2014); however, even with treatment, mortality rates can be as high as 70% (Kyvernitakis et al., 2016).

Iron is an essential nutrient for both the host and pathogen as it is required for many biological processes including DNA synthesis and cellular respiration (Haas, 2003). To limit the growth of pathogens *in vivo*, serum contains iron-binding proteins such as transferrin and lactoferrin that maintain a very low concentration of free iron ($\sim 10^{-18}$ M). To overcome this iron limitation, microbes employ numerous strategies, including reductive iron assimilation (RIA) and

Abbreviations: CAS, chrome azurol S; DKA, diabetic ketoacidosis; NIS, NRPS-independent siderophore; NRPS, non-ribosomal peptide synthetase

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uptake by a high-affinity Fe^{2+} transporter (Hissen et al., 2004; Li et al., 2016). In *R. delemar*, reduced expression of the high affinity iron permease, FTR1, resulted in attenuated infection and reduced mortality in DKA mice (Ibrahim et al., 2010). An alternative mechanism of iron uptake involves the biosynthesis and secretion of siderophores, low molecular weight organic molecules that are released into the environment under iron-limiting conditions to chelate ferric iron. Hydroxamate siderophores (named for the moiety that complexes ferric iron) are the major class produced by non-Mucorales fungi. In contrast, fungi within the order Mucorales have been shown to produce the polycarboxylate siderophore rhizoferrin (Drechsel et al., 1991; Thieken and Winkelmann, 1992). The chemical structure of rhizoferrin consists of a diaminobutane backbone linked to two citric acid moieties, with an *R,R*- configuration around the chiral center (Fig. 1A) (Drechsel et al., 1992). Rhizoferrin is also produced by *Francisella tularensis* and *Ralstonia* (*Pseudomonas*) *pickettii* (Sullivan et al., 2006; Taraz et al., 1999). The two siderophores have the same molecular formula; however, bacterial rhizoferrin is an enantiomer of fungal rhizoferrin with an *S,S* configuration around the chiral center (Sullivan et al., 2006; Taraz et al., 1999). While the structure of *R,R*-rhizoferrin has been elucidated, little is known about its biosynthesis in Mucorales.

The biosynthesis of siderophores is known to occur via two main pathways. The first involves non-ribosomal peptide synthetases (NRPS) that catalyze the condensation of multiple amino acids to form siderophores. The second relies on an NRPS-independent siderophore (NIS) pathway. NIS enzymes function by adenylating a substrate carboxyl group for subsequent condensation with a polyamine or amino alcohol (Gulick, 2009). At present, there are four proposed classes for NIS enzymes based on their substrate specificity for polyamines or amino alcohols and which substrate is activated for downstream condensation (Cotton et al., 2009; Oves-Costales et al., 2009). Type A NIS synthetases catalyze condensation of citric acid with various amines and alcohols. The Type A' NIS synthetase sub category catalyzes condensation of citric acid specifically with amines. Type B NIS synthetases are predicted to catalyze condensation of α-ketoglutarate with amines; however, only one such enzyme has been biochemically characterized (Kadi and Challis, 2009). Type C NIS synthetases are specific for monoamine or monoester derivatives of citric acid, or monohydroxamate derivatives of succinic acid, and some type C NIS synthetases are known to catalyze formation of oligomeric/macrocyclic siderophores (Oves-Costales et al., 2009).

The NIS enzymes, SfnaD and SfnaB are responsible for biosynthesis of the polycarboxylate siderophore, staphyloferrin A (Fig. 1B) in the bacterial pathogen, *Staphylococcus aureus*. In *S. aureus,* SfnaD condenses one molecule of citric acid to D-ornithine forming a citryl-ornithine intermediate which is then used as substrate by SfnaB to condense a second molecule of citrate, yielding the final product staphyloferrin A (Cotton et al., 2009). Unlike staphyloferrin A, rhizoferrin is a symmetric molecule; therefore, we hypothesized that the synthesis of rhizoferrin should require only a single Sfna-like enzyme to catalyze the condensation of two molecules of citrate to one molecule of diaminobutane in sequential reactions.

2. Materials and methods

2.1. Bioinformatic analyses

Bioinformatic analyses were conducted using the genomic sequence of *Rhizopus delemar* (99–880) published by Ibrahim et al. (2009); and acquired from Genbank (accession: PRJNA13066). Genetic information about *rfs* (accession: RO3G_06864) was obtained from NCBI. Conserved domains were identified in the Rfs protein sequence using the NCBI Conserved Domain Database (Marchler-Bauer et al., 2015) and Blastp was used to identify Rfs homologs (Altschul et al., 1990). Phyre and I-TASSER (Roy et al., 2011; Kelley et al., 2015) were used to predict the structure of Rfs using the crystal structure of AcsD as a template (PDB: 2W02). PyMOL was used for structural alignments and to produce all protein structure figures (The PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC).

Phylogenetic analyses were carried out on NIS synthetases using a Clustal Omega multiple sequence alignment (Sievers et al., 2011). NIS enzymes used for the alignment are listed in the supplementary information. Analysis of the multiple sequence alignment was done using Jalview (version 2.10.1; Waterhouse et al., 2009). Phylogenetic analyses were completed using Geneious (version 9.1.5; Kearse et al., 2012) and a Neighbor-Joining consensus tree was made. Bootstrap values are indicated at branch nodes.

2.2. Strains, media and culture conditions

Rhizopus delemar 99–880 was obtained from American Type Culture Collection (ATCC). *Mucor circinelloides* (UAMH 8307), *Lichtheimia corymbifera* (UAMH 10324) and *Rhizomucor pusillus* (UAMH 10076) were obtained from the University of Alberta Microfungus Collection and Herbarium. *Syncephalastrum racemosum*, *Cunninghamella echinulata* and *Mucor heimalis* were obtained from the culture collection at Simon Fraser University (Burnaby, Canada). To induce siderophore expression, fungi were grown in low iron media, Media A (20 g sucrose, 3 g $(NH_4)_2SO_4$, 1 g K_2HPO_4, 1 g KH_2PO_4, 25 mg $MgSO_4{\cdot}7H_2O$, 10 mg $CaCl_2{\cdot}2H_2O$, 0.2 mg $ZnSO_4{\cdot}7H_2O$, 1 l water) or Eagle's Minimum Essential Medium (MEM; Sigma) + 10% human serum (male AB positive; Sigma). *Escherichia coli* DH5α was used in standard cloning procedures. Tuner *E. coli* was used for protein expression. Both *E. coli* strains were grown on Luria-Bertani (LB) media (10 g tryptone, 10 g NaCl, 5 g yeast extract, 1 l water) supplemented with ampicillin (100 μg/ml) or kanamycin (30 μg/ml) as needed. All transformations were done using heat-shock and *E. coli* cells were made competent via incubation in 100 mM $CaCl_2$ or in some cases *E. coli* cells were made ultra-competent (Sambrook and Russell, 2006).

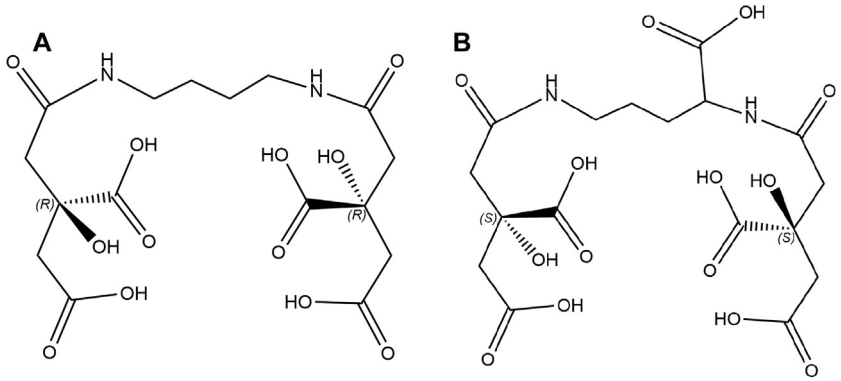

Fig. 1. The chemical structures of (A) *R,R*-rhizoferrin and (B) *S,S*-staphyloferrin.