FUNGAL BIOLOGY XXX (2015) 1–6

British Mycological Society promoting fungal science

journal homepage: www.elsevier.com/locate/funbio

Regulation of the aflatoxin-like toxin dothistromin by AflJ

Pranav CHETTRI[a], *Kenneth C. EHRLICH*[b], *Rosie E. BRADSHAW*[a,*]

[a]Bio-Protection Research Centre, Institute of Fundamental Sciences, Massey University, Palmerston North 4442, New Zealand

[b]Southern Regional Research Center, Agricultural Research Service, USDA, New Orleans, LA 70124, USA

ARTICLE INFO

Article history:
Received 7 November 2014
Received in revised form
28 January 2015
Accepted 30 January 2015
Corresponding Editor:
Geoff M. Gadd

Keywords:
Gene regulation
Mycotoxin
Polyketide
Secondary metabolite

ABSTRACT

Biosynthesis by *Aspergillus parasiticus* of aflatoxin, one of the most potent known naturally occurring carcinogens, requires the activity of two regulatory proteins, AflR and AflJ, which are encoded by divergently transcribed genes within the aflatoxin gene cluster. Although the Zn_2Cys_6 transcription factor, AflR, has been well-studied, the role of AflJ as a transcription regulatory factor is not well understood. An AflJ-like gene (*DsAflJ*) is also present in the genome of the pine needle pathogen *Dothistroma septosporum* and is similarly divergently transcribed from an AflR orthologue (*DsAflR*). These genes are involved in biosynthesis of dothistromin, a toxic virulence factor related to aflatoxin. *DsAflJ* mutants produced low levels of dothistromin (<25-fold less than wild-type); this was in contrast to earlier work with *A. parasiticus AflJ* mutants in which aflatoxin production was more severely impaired. As expected, complementation of *D. septosporum* mutants with an intact copy of the *DsAflJ* gene regained production of wild-type levels of dothistromin, although levels were not further increased by over-expression in multi-copy strains. However, heterologous *AflJ* genes from *Aspergillus* spp. were unable to complement *DsAflJ* mutants, suggesting that the proteins function differently in these species.

Introduction

Many filamentous fungi make potent toxins, such as aflatoxin, that cause acute problems for humans and livestock (Gnonlonfin *et al.* 2013). For this reason, considerable research effort has been devoted to understanding how the biosynthesis of toxins such as aflatoxin is regulated, in the hope of curbing their production (Yu & Ehrlich 2011). One well-studied regulatory protein is AflR, a transcription factor that co-regulates expression of most aflatoxin biosynthetic genes in several species of *Aspergillus* (Price *et al.* 2006). In contrast relatively little is known about another putative aflatoxin regulatory gene, *AflJ* (also called *AflS* in *Aspergillus* spp.), which is adjacent to the *AflR* gene and divergently transcribed (Yu *et al.* 2004). Previous studies suggested that, like AflR, AflJ acts as a positive regulator of clustered aflatoxin and sterigmatocystin genes (Meyers *et al.* 1998; Chang 2003), and it was suggested that AflJ serves as a transcriptional co-activator. More recently, another study found that although AflJ binds to AflR it may serve as a chaperone of AflR and affect its cellular

* *Corresponding author*. Institute of Fundamental Sciences, Massey University, Manawatu Campus, Palmerston North 4442, New Zealand. Tel.: +64 6 356 9099x84735; fax: +64 6 350 5682.
E-mail addresses: p.chettri@massey.ac.nz (P. Chettri), ehrlich8@gmail.com (K. C. Ehrlich), r.e.bradshaw@massey.ac.nz (R. E. Bradshaw).

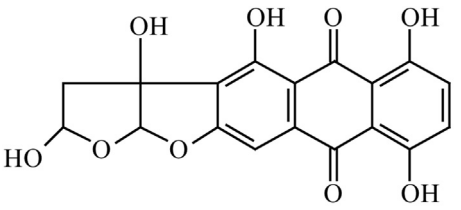
http://dx.doi.org/10.1016/j.funbio.2015.01.007
1878-6146/

Please cite this article in press as: Chettri P, et al., Regulation of the aflatoxin-like toxin dothistromin by AflJ, Fungal Biology (2015), http://dx.doi.org/10.1016/j.funbio.2015.01.007

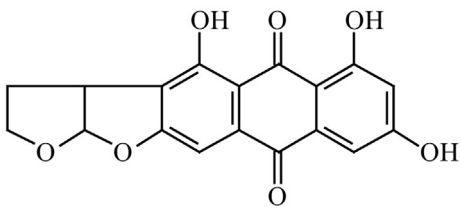
stability and intracellular transport (Ehrlich *et al.* 2012). AflJ-like proteins appear to have no significant domain similarity with any known enzymatic or regulatory proteins and do not have DNA-binding domains, although the *Aspergillus flavus* and *Aspergillus parasiticus* AflJ proteins are predicted to have three putative membrane-spanning helices and a microbody targeting signal (Meyers *et al.* 1998; Ehrlich *et al.* 2011).

The forest pathogen *Dothistroma septosporum* also has an *AflJ*-like gene (*DsAflJ*) thought to be associated with biosynthesis of the virulence factor dothistromin, a chemical relative of aflatoxin (Fig 1) (Chettri *et al.* 2013; Kabir *et al.* 2014). Dothistromin biosynthetic genes are arranged at six separate loci on a 1.3-Mb chromosome (Chettri *et al.* 2013) rather than being clustered as in aflatoxin biosynthesis (Keller & Hohn 1997). But despite their dispersal, expression of dothistromin genes is co-regulated by DsAflR, an orthologue of the aflatoxin pathway-specific regulator AflR. The presence of a putative AflJ interaction motif at the C terminal of DsAflR (Chettri *et al.* 2013) suggests a similar close relationship between DsAflR and DsAflJ in *D. septosporum* as is seen in *Aspergillus* spp. In *D. septosporum* the *DsAflJ* gene is also adjacent to *AflR* and divergently transcribed, although with a longer intergenic region (2.3 kb; Chettri *et al.* 2013) compared to the corresponding region in *A. parasiticus* (0.74 kb; Ehrlich *et al.* 2011). This divergent arrangement is conserved in other fungi with AflR- and AflJ-like genes (Bradshaw *et al.* 2013), suggesting a selective advantage of having both regulatory genes together.

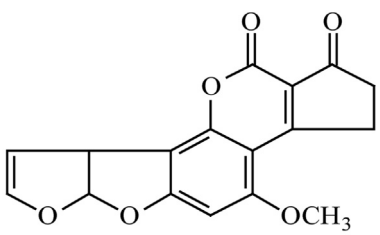
Given the importance of aflatoxin-type toxins and the occurrence of *DsAflJ*-like genes in a phylogenetically broad range of toxigenic fungi, more information is needed on the role of AflJ. Thus we tested the hypothesis that DsAflJ is required for dothistromin biosynthesis and determined whether *AflJ* genes from aflatoxigenic species could fulfill the function of DsAflJ in *D. septosporum*.



Dothistromin



Versicolorin B



Aflatoxin B1

Fig 1 – Chemical structures of dothistromin, the aflatoxin precursor versicolorin B, and aflatoxin B1.

Methods

Production of DsAflJ *gene knockout mutants*

To elucidate the role of DsAflJ in dothistromin production, gene replacement and complementation mutants were made in *Dothistroma septosporum*. *DsAflJ* mutants were prepared by homologous recombination in which the entire *DsAflJ* gene was replaced by a hygromycin resistance (*hph*) gene ensuring no portion of the DsAflJ protein was produced by the mutants. The *DsAflJ* gene replacement construct was made using a Gateway (Invitrogen, CA, USA) cloning strategy as described previously (Zhang *et al.* 2007). Three entry clones were created by PCR amplification of a 1.1 kb 5′ region of *DsAflJ* (JGI protein ID 57214), a selectable marker that confers hygromycin resistance (*hph*) and a 1.15 kb 3′ region of *DsAflJ*, then combined into a three-fragment plasmid pR316. The hygromycin gene was under the control of the *Aspergillus niger glaA* promoter and the *Aspergillus nidulans trpC* terminator. Primer sequences are shown in Supplementary Material, Table S1. Wild-type (WT) *D. septosporum* strain NZE10 was transformed with pR316 and targeted replacement of *DsAflJ* confirmed by PCR and Southern hybridisation using methods described previously (Zhang *et al.* 2007) (Supplementary Material, Fig S1).

Transformation of mutants with homologous or heterologous AflJ *genes*

DsAflJ knockout mutant strain FJT112 was transformed with intact copies of *DsAflJ*, or with *AflJ* homologues from *Aspergillus parasiticus* (AAS66019.1), *Aspergillus nidulans* (*MdpA*; ANID_10021) (Chiang *et al.* 2010) or *Cladosporium fulvum* (*CfAflJ;* JGI protein ID 197013) to determine if the homologues were able to complement the mutant phenotype. *CfAflJ* was included because *C. fulvum* is closely related to *Dothistroma septosporum* and contains orthologs of dothistromin genes, although some of them are non-functional (Chettri *et al.* 2013). The *MdpA* gene was included as it is more similar to DsAflJ (41.4 % amino acid identity) than the *A. nidulans* sterigmatocystin AflJ is to DsAflJ (36 % amino acid identity). For *DsAflJ*, a complementation vector was constructed by amplifying 1485 bp of the complete *DsAflJ* coding region along with 900 bp of upstream and 1017 bp of downstream gDNA sequence using Platinum High Fidelity polymerase (Invitrogen, CA, USA) and primers 5′ *ApaI* and 3′ *XbaI* (Supplementary Table 1). The PCR product was ligated into pBC-phleo (Chettri *et al.* 2012) to create *DsAflJ* complementation plasmid pR317. For transformation with heterologous genes, the coding regions and ~1 kb of 5′ and 3′ flanking sequence of *AflJ* or *AflJ*-like genes from the other species listed above were amplified and ligated into a pBC-phleo vector as before. Complementation plasmids were transformed into *ΔDsAflJ* FJT112 and

Please cite this article in press as: Chettri P, et al., Regulation of the aflatoxin-like toxin dothistromin by AflJ, Fungal Biology (2015), http://dx.doi.org/10.1016/j.funbio.2015.01.007