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Lichen ketosynthase domains are not responsible for inoperative polyketide synthases in *Ascomycota* hosts

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ABSTRACT

Efforts by lichenologists to characterize lichen polyketide synthases (PKS) through heterologous expression experiments have so far proved unfruitful. A determination of systematic causes of failure is therefore required. Three hypotheses involving the ketosynthase (KS) domain of lichen polyketide synthases (PKS) from *Cladonia uncialis* are tested: (1) Horizontal versus vertical gene transfer; (2) Typical versus atypical active site residues; (3) Typical versus atypical tertiary protein structure and active site architecture. Phylogenetics, amino acid sequence alignment, and protein modelling indicate that *C. uncialis* PKS evolved through vertical transfer from *Ascomycota* fungi, possess Cys-His-His catalytic triads typical of KS from most organisms, and possess protein and catalytic site architecture identical to well-characterized KS from non-lichen organisms. Though the reason for lack of functional activity in heterologous hosts remains unknown, complications involving the KS are ruled out as a likely explanation. Heterologous translation of lichen PKS (or parts thereof) have not been reported. We demonstrate heterologous translation of two lichen KS domains in *E. coli*.

1. Introduction

Lichens are fungi that form symbiotic relationships with algae and cyanobacteria [1]. More than one thousand secondary metabolites have been isolated from lichenizing fungi [2]. Lichens are adept at thriving in nutrient-minimal environments and can have lifespans extending thousands of years [3]. Biomass accumulation in the laboratory proceeds at a rate of one centimeter diameter expansion per year [4]. For this reason, otherwise routine tasks such as isolating DNA from sub-cultured specimens or conducting time-course experiments are challenging. Though recent genome sequencing work has revealed the genetically encoded potential for natural product production in lichens [5,6], the biosynthetic processes associated with known secondary metabolites remain mysterious. Polyketide synthases (PKS) are a class of biosynthetic enzymes present in lichenizing and non-lichenizing fungi, plants, and bacteria [7]. Several well-established fungal platforms are available to characterize PKS genes through functional heterologous expression experiments [8–10]. Using these platforms, several independent research groups have attempted to characterize PKS genes from lichenizing fungi. Though in all cases transcription including intron splicing has been observed in *Ascomycota* platforms, in no trial was *de novo* production of a polyketide observed [11–14]. We have observed similar results in experiments involving two PKS from *Cladonia uncialis* [15,16]. The fact that multiple independent groups employing distinct methods have failed to functionally express lichen PKS suggests that an unknown systematic problem exists. Attention is now shifting towards investigating why these various attempts have failed to produce functional PKS in heterologous hosts.

The ketosynthase (KS) domain of PKS catalyses the formation of carbon-carbon bonds, the key chemical reaction involved in all polyketide chemistry [17]. Functions for two PKS in the lichen *Cladonia uncialis* have been proposed: A putative methylphloroacetophenone synthase (MPAS) responsible for usnic acid biosynthesis [15] and a putative 6-hydroxymellein synthase (6HMS) associated with an unknown halogenated isocoumarin [16]. Evolutionary, mechanistic, and structural hypotheses explaining absence of function in *Ascomycota* hosts were tested on the KS domains of MPAS and 6HMS. These tests were unable to reveal evolutionary, structural, or mechanistic abnormalities indicative of a cause of these failures. Although at present it

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remains unknown why lichen PKS cannot be functionally expressed, we can now rule out complications involving the KS domain as a plausible explanation.

2. Materials and methods

2.1. Phylogenetics, sequence alignment, and protein modelling

Amino acid sequences of three representative KS domains from each of the 14 sub-families of the KS3 family were selected at random for phylogenetic analysis [18]. The KS of *Arabidopsis thaliana* (NP_179113), a member of the KS2 family, was chosen as an outgroup. Multiple sequence alignment was performed using Clustal W [19]. Phylogenetic analysis was performed using MEGA7 [20]. The neighbour-joining method was used to infer evolutionary history [21]. The resultant tree was evaluated using 1000 replicates of the bootstrap test [22]. The estimated evolutionary distance was determined using the Poisson correction method [23]. Protein modelling performed with Swiss Model [24], freely available at https://swissmodel.expasy.org/. Models were processed with DeepView/Swiss-PdbViewer (V.4.1) [25], freely available at https://spdbv.vital-it.ch/.

2.2. Cloning and expression of KS domains

The taxonomic identification of *Cladonia uncialis*, mycobiont sub-culturing, genome sequencing, and gene annotation have been previously described [5,6,15,16]. The KS domains of MPAS (ALA62323) and 6HMS (ANM27730) were amplified by PCR using 30 cycles of denaturation (94°C | 30s), annealing (56°C | 15s) and extension (65°C | 240s), including an initial denaturation phase (95°C | 120s) and final extension phase (65 °C | 600s). Primers: MPAS-KS-F (CGCGAACAGATTGGAGGTGACCCTTTTGGATCGGTTTC), MPAS-KS-R (GTGGCGGCCGCTCTATTAATTGATGCAAGCGGCTAATC), 6HMS-KS-F (CGCGAACAGATTGGAGGTTGTACTCACTGCGGTCGTCT), 6HMS-KS-R (GTGGCGGCCGCTCTATTAGTATGCTCGCCTCTTTCCAC). Amplicons were purified from agarose using the GeneJet Gel Extraction Kit (ThermoFisher). Purified amplicons were ligated into pETite vector using the Expresso T7 SUMO Cloning and Expression System (Lucigen), and plasmids transformed into BL21 *E. coli*. Expression of KS proteins was achieved by growing cultures in LB media to OD_{600} of 0.6–0.7, adding IPTG (1 mM), incubating for two hours (37°C | 250 rpm) followed by eight hours (19°C | 150 rpm). Total protein was extracted using sonication and the B-PER 6xHis Fusion Protein Purification Kit (Pierce). The His(6)-SUMO-tagged KS were purified using a gravity nickel column with imidazole as the elution buffer. The purified proteins were electrophoresed on 12% SDSPAGE to confirm protein size as compared to molecular weight standards.

2.3. Mass spectrometry

Protein bands were purified from SDSPAGE gels according to described methods [26]. Purified proteins (1 mg/mL) were reduced with dithiothreitol (10 mM) and cysteine residues were alkylated with iodoacetamide (55 mM). After dialysis into 100 mM NH_4HCO_3, TPCK-treated trypsin was added to 0.01 mg/mL, and samples incubated overnight at 37 °C. One microliter volumes of protein and DHB matrix (2,5-dihydroxybenzoic acid in 50% methanol and 2% formic acid) were spotted onto a custom metal target. Spectra were obtained using a prototype MALDI QqTOF instrument built in the University of Manitoba [27]. Spectra were analysed by non-commercial software developed with the instruments, and ions were compared to those expected from a virtual digest of the putative protein sequence determined from the mRNA sequences of *mpas* and 6 hms from *Cladonia uncialis* [15,16]. Some ions were chosen for tandem mass spectrometry, and their fragmentation patterns were compared to those expected from virtually-digested peptides.

3. Results and discussion

3.1. Phylogenetic analysis of KS domains

Lichens are not merely symbionts of fungi and algae but micro-communities encompassing dozens of cohabitating organisms [28–30]. Lichenizing fungi have also displayed a propensity during their evolutionary history for switching algal partners [31]. Although vertical gene transfers predominate the evolutionary history of lichens, horizontal gene transfers have also shaped the evolution of the secondary metabolome [32–34]. Although *Cladonia uncialis* is taxonomically classified under phylum *Ascomycota*, it is possible that *mpas* and 6 hms arose through horizontal gene transfer. It is therefore possible that *mpas* and 6 hms do not originate from an *Ascomycota* lineage even though *C. uncialis* is an *Ascomycota* fungus. This distinction, if true, could explain why *Ascomycota* hosts are unable to functionally express lichen PKS [11–16].

The KS is the most evolutionarily conserved PKS domain and is therefore used to study the evolution of PKS of lichenizing fungi [35]. We therefore used the KS of *mpas* and 6 hms to test the hypothesis that either PKS has an evolutionary origin beyond *Ascomycota*. Ketosynthases, a broad term for enzymes catalyzing condensations of acyl-coenzyme A and related substrates, have been classified into five families [18]. The third family, KS3, is composed of modular and iterative PKS as well as fatty acid synthases. The KS3 family is sub-divided into 14 groups (Named '3A' to '3 N') comprising both prokaryotes and eukaryotes [18]. Three representative KS domains of each of these 14 groups were selected randomly and a phylogenetic tree was constructed including the KS of *mpas* and 6 hms. The expected outcome was that KS of *mpas* and 6 hms would cluster within one of the 14 groups indicating a phylogenetic relationship. This experiment revealed *mpas* and 6 hms to cluster within group '3 M', comprising *Ascomycota* (Fig. 1). These results suggest that *mpas* and 6 hms are indeed *Ascomycota* PKS genes. The hypothesis that these PKS were not *Ascomycota*-inherited genes was therefore invalidated.

3.2. Multiple sequence alignment of KS domains

Decarboxylative Claisen condensation by KS is performed by a Cys-His-His catalytic triad. These residues are found within conserved TACSSS, EHGTGT, and KSNIGH motifs [36]. Mutational and biochemical analyses indicate that the purpose of histidine (EHGTGT) is to facilitate the transfer of the ACP-polyacyl intermediate to cysteine by acidifying the ACP-thioester intermediate [37]. The other histidine (KSNIGH) supports this transfer by serving as a general base to increase the nucleophilicity of the cysteine thiol [37]. These residues also support decarboxylation of methylmalonyl-ACP and C-C bond formation by transition state stabilization and stereoselective orientation of the substrates. The purpose of cysteine (TACSSS) is to provide a nucleophilic anchor for polyketide intermediates [37].

Though this Cys-His-His triad is highly conserved, it is not absolutely required for polyketide assembly to occur nor are ketosynthases restricted to C-C bond formation as a biological role [38]. What residues are present may therefore have broad functional implications. For example, chalcone synthases (Type III PKS) and some fatty acid synthases employ Cys-His-Asn triads [39,40]. Whereas type I PKS recruit acetyl-CoA primer via AT-ACP loading

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