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Comparison of 10,11-dehydrocurvularin polyketide synthases from *Alternaria cinerariae* and *Aspergillus terreus* highlights key structural motifs

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Abstract

Iterative type I polyketide synthases (PKSs) from fungi are multifunctional enzymes that use their active sites repeatedly in a highly ordered sequence to assemble complex natural products. A phytotoxic macrolide with anticancer properties, 10,11-dehydrocurvularin (DHC), is produced by cooperation of a highly reducing (HR) iterative PKS and a non-reducing (NR) iterative PKS. We have identified the DHC gene cluster in *Alternaria cinerariae*, heterologously expressed the active HR PKS (Dhc3) and NR PKS (Dhc5) in yeast and compared them to corresponding proteins that make DHC in *Aspergillus terreus*. Phylogenetic analysis, and homology modeling of these enzymes has identified variable surfaces and conserved motifs that are implicated in product formation.

Graphical Abstract

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Supporting information for this article is given via a link at the end of the document.

We have identified and heterologously expressed two iterative type I PKSs necessary for production of the phytotoxic anticancer agent 10,11-dehydrocurvularin (DHC) in *Alternaria cinerariae*. This afforded the unique opportunity for bioinformatic comparison to the analogous metabolite gene cluster in *Aspergillus terreus*, which highlighted key structural features necessary for metabolite production.

Keywords

bioinformatics; biosynthesis; dehydrocurvularin; heterologous expression; polyketide

Curvularins are a class of phytotoxins produced by several species of fungi including *Alternaria*, *Nectria*, *Curvularia*, *Penicillium*, *Aspergillus* and *Eupenicillium*.^[1–10] These compounds contain a 12-membered dihydroxyphenylacetic acid lactone (DAL) ring, similar to the 14-membered resorcylic acid lactone (RAL) ring found in polyketides such as hypothemycin and radicicol (Figure 2), previously investigated by our groups.^[11,12] The curvularins inhibit the inducible nitric oxide synthase (iNOS) in mammals, thus modulating the proinflammatory immune response.^[7] 10,11-Dehydrocurvularin (DHC) (1) induces overexpression of heat shock factor 1 (HSF1) and various other chaperone proteins, thereby activating the heat shock response, a conserved evolutionary mechanism by which cells maintain protein homeostasis.^[13] Additionally, DHC and curvularin inhibit the TGF- β signalling pathway, which is upregulated during tumor progression.^[14] Via these two mechanisms, DHC acts as a broad-spectrum inhibitor of several cancer cell lines *in vitro*, with the potential for development as a new cancer therapeutic.^[8] These effects and other biological activities have prompted a number of chemical syntheses of curvularin derivatives.^[15–22]

After elucidation of the structure of DHC, Arthur Birch and colleagues chose this molecule as one of the first for classical biosynthetic studies on polyketides.^[23] Our group subsequently confirmed the origin of all constituent atoms and successfully incorporated advanced isotopically labeled intermediates into DHC using cultures of *Alternaria cinerariae*.^[24–27] Recently, the heterologous expression of two PKSs (CurS1 and CurS2) responsible for DHC production in *Aspergillus terreus* was reported by Molnar and

coworkers.^[28] They showed that in *A. terreus*, DHC is biosynthesized by cooperation of two type I iterative polyketide synthases (PKSs)^[29–31] - a highly reducing (HR) PKS and a non-reducing (NR) PKS - in a similar fashion to RALs. The NR PKS CurS2 was also re-engineered in its product template (PT) domain to make RALs instead of the DAL dehydrocurvularin.^[32] Formation of the same secondary metabolite, DHC (1), by two quite different fungal classes, namely Dothideomycetes (*Alternaria*) and Eurotiomycetes (*Aspergillus*) provides a unique opportunity to compare biosynthetic machinery for its production. We now report that despite similar enzymatic machinery (ca. 75% identity) and highly conserved active sites, certain regions and surfaces of corresponding PKS protein domains are quite different. With the goal of complete reconstitution of DHC PKSs via heterologous expression in *Saccharomyces cerevisiae*, we sent the genome of *Alternaria cinerariae* (ATCC 11784) for Illumina Sequencing (Ambry Genetics). A total of 34Mb of genomic information was obtained over a total of 907 contigs. The genome was annotated using Antibiotics & Secondary Metabolite Analysis Shell (antiSMASH v. 2.0).^[33] A total of 39 secondary metabolite genes were identified in the genome of *A. cinerariae*, 8 of which are for type I iterative PKSs. Only one gene cluster closely resembles the gene clusters of hypothemycin and radicicol. Hidden Markov model (HMM)-based gene sequences contained within this gene cluster were predicted with FGENESH (Softberry),^[34] and the resulting gene sequences analyzed individually using BLAST (NCBI). Five genes potentially related to DHC biosynthesis were identified (Figure 1).

Both intron-free *dhc3* (7.1 kb) and *dhc5* (6.2 kb) were cloned into plasmids for expression in *S. cerevisiae* BJ5464-NpgA and subsequently used for single and double transformations. Both Dhc3 and Dhc5 could be isolated from single transformants, and neither produced DHC alone. Double transformation yielded on average 11 mg/L of DHC after RP-HPLC purification (Supporting Information Figure S1). The identity of DHC was confirmed by NMR, and by LC-ESI-MS using combined retention time matching with accurate mass matching (Supporting Information Figures S2–S4). This confirmed Dhc3 and Dhc5 to be responsible for DHC biosynthesis in *A. cinerariae*. With the aim of comparing the production of DHC in different organisms, we embarked on genetic sequence and structural studies.

Alternaria and *Aspergillus* share a common ancestor in Ascomycota. However, *Alternaria* belongs to the Dothideomycetes fungal class (11 orders, 90 families, 1300 genera, 19,000 species)^[35] whereas *Aspergillus* is in the less well-defined Eurotiomycetes class. We prepared a phylogenetic tree of Dhc3 and Dhc5 in comparison to some of their top BLAST hits that produce either DAL or RAL polyketides (Figure 2 and Supporting Information Figure S4). Phylogenetic analysis revealed that even those organisms that produce DAL or RAL type polyketides appear relatively far away from one another in terms of common ancestors. Considering the early evolutionary point at which *Alternaria* and *Aspergillus* diverge, it is remarkable that these two organisms make the same natural product using similar machinery.

We then specifically compared sequences of Dhc3 and Dhc5 to the recently published sequences of the DHC producing PKSs from *A. terreus*, CurS1 and CurS2.^[28] Although Dhc3 and Dhc5 share 75% and 77% sequence identity with CurS1 and CurS2, respectively,

the differences appear to be clustered in specific areas. To investigate the specific effect of this nature-induced mass mutation experiment, we aligned each separate domain of the Dhc3 sequence with that of CurS1, and Dhc5 with CurS2 (Table 1). The pseudo KR domain (or structural KR subdomain) found in the HR PKSs contains a truncated Rossmann fold and acts to stabilize the catalytic subdomain. Comparing Dhc3 and CurS1, the standout difference is this domain. It therefore appears from our analysis that the sequence of these sub-domains has little influence on the outcome of dehydrocurvularin biosynthesis. Further mutation or partial deletion of these domains may elaborate upon their necessity for metabolite production. For Dhc5, the 23% difference when compared to CurS2 is spread out over the entire protein, concentrated mainly in inter-domain linking sequences as expected. The least homologous domain between CurS2 and Dhc5 is the SAT domain, with 71% sequence identity. This is to some extent expected, as the SAT domain of NR PKSs forms specific protein-protein interactions when transferring the polyketide intermediate from the HR PKS partner.^[11] Thus, this protein-protein interaction appears to differ significantly between different organisms.

To investigate the structural differences between the two sets of proteins further, we prepared homology models of selected protein domains, namely the AT domain of Dhc3 (AT_{Dhc3}) and the PT domain of Dhc5 (PT_{Dhc5}), using I-TASSER.^[36–38] We selected AT_{Dhc3} based on its low sequence identity to CurS1 and the availability of a crystal structure of an iterative type I HR PKS domain, DynE8 (AT_{DYN10}) from *Micromonospora chersina*.^[39] Also, a crystal structure of the PT domain from the iterative NR PKS PksA, responsible for biosynthesis of aflatoxin B₁ in *Aspergillus parasiticus*, was recently published,^[40] making PT_{Dhc5} a good candidate for modeling. Both of these domains are involved in intermediate recognition, and we hoped that homology modeling might illuminate potential structural similarities key for substrate recognition.

Using the AT homology model, we mapped “mutations” of AT_{Dhc3} found in the CurS1 sequence. The results show that the differences exist almost exclusively on the surface, and primarily on one face of the protein, Figure 3. Electrostatic surface maps of AT_{Dhc3} and AT_{CurS1} homology models highlighted that the anionic surface around the active site remains the same. However, the cationic surface of AT_{CurS1} is somewhat more diffuse in comparison to AT_{Dhc3} (Supporting Information, Figure S6 and S7).

Notably, PT_{Dhc5} includes the conserved W₁₅₈₃ (CurS2 W₁₅₈₄), which acts as a “gate-keeping” residue near the PT substrate entrance channel, whereby it directs the C2 (see Figure 1) of the substrate away from the catalytic histidine.^[32] Also F₁₄₅₄ (CurS2 F₁₄₅₅) and Y₁₅₇₅ (CurS2 Y₁₅₇₆) are maintained in Dhc3 PT. These residues are located at the back of the substrate binding pocket, where they participate in electrostatic H-bonding, or hydrophobic interactions with the substrate, respectively, thereby directing DAL or RAL formation.^[32] Interestingly however, the active site cavities vary quite significantly in overall structure, Figure 4. Although the aforementioned amino acids remain structurally conserved, the substrate cavity of PT_{CurS2} extends far beyond the cyclization chamber. CurS2 has a long hydrophobic chamber attached; perhaps indicating relaxed substrate specificity and increased enzyme promiscuity.

Recently, the crystal structure of the SAT domain of CazM, the NR PKS involved in the biosynthesis of chaetoviridin A and chaetomugilin A in *Chaetomium globosum*, was determined.^[41] A key structural feature of SAT_{CazM} is a “parallel” α P helix contained within the α/β -hydrolase-like subdomain (Supporting Information Figure S9) that is thought to be important in ACP-SAT interactions. We modeled SAT_{Dhc5} and SAT_{CurS2} based on this sequence and also without any structural constraints (Figure 5 and Supporting Information Figures S11 and S12). We found that in both cases, the α P helix of SAT_{Dhc5} and SAT_{CurS2} is predicted to be in a “perpendicular” orientation, consistent with all known AT domain crystal structures, most of which are from modular PKSs.

Furthermore, our phylogenetic analysis of NR PKS SAT domains (Supporting Information Figure S13) shows that Dhc5 and CurS2, along with all other DAL and RAL forming PKSs, clade closer to the AT3 domain from the type I iterative modular PKS, 6-deoxyerythronolide B synthase (DEBS), than that of CazM. Based on this knowledge, it may be reasonable to suggest that the SAT domains from DAL and RAL forming NR PKSs evolved directly from their AT relatives, which also contain an active site serine.

Heterologous expression of the HR PKS and NR PKS megasynthase pair Dhc3 and Dhc5, respectively, confirmed the identity of the putative DHC gene cluster from *A. cinerariae*. Based on our analyses the sequence differences between the AT domains of Dhc3 and CurS1 are found mainly on the surface of the protein. For Dhc5, the SAT domain showed the lowest homology with that of CurS2. This is in line with previous thinking that this domain is highly specific for each organism. Furthermore, the PT domain of Dhc5 contains conserved residues that had previously been suggested to lead to DAL ring formation over RAL ring formation. As it is well understood that single point mutations can abolish enzyme activity, a remarkable outcome of the present study is that substantial (23–25%) natural alterations of protein sequence in these megasynthases still results in fully active enzymes that produce significant amounts of DHC with high fidelity.

Experimental Section

Genomic DNA Extraction and Sequencing

Genomic DNA (gDNA) was first isolated from a 7-day culture of *Alternaria cinerariae* (ATCC 11784). The mycelia were flash frozen in liquid nitrogen and ground in a mortar and pestle. gDNA was then extracted with the DNeasy Plant Mini Kit (Catalog no. 69104, Qiagen) and sent to Ambry Genetics for sequencing using Illumina HiSeq 2000 technology. GenBank® Accession numbers KT271470, KT271471, KT271472, KT271473, and KT271474 (Dhc1-5, respectively).

Molecular cloning of Dhc3 and Dhc5

Two 2 μ -based yeast-*E. coli* shuttle vectors, pKOS518-120A and pXK30 were used for expression of Dhc3 and Dhc5. pKOS518-120A contains the *TRP1* auxotrophic marker and *NdeI-EcoRI* restriction endonuclease sites. pXK30 contains the *URA3* auxotrophic marker and *NdeI-PmlI* restriction endonuclease sites. Total RNA was first isolated from a 7-day culture of *Alternaria cinerariae* (ATCC 11784) using the RNeasy Plant Mini Kit (Catalog no. 74903, Qiagen), and converted into complementary DNA (cDNA) using AccuScript

HighFidelity 1st Strand cDNA Synthesis Kit (Catalog no. 200820, Agilent). Primers containing a 35 bp overlap with the ADH2 promoter and terminator sequences of pXK30 and pKOS518-120A were ordered from Integrated DNA Technologies and used to amplify Dhc3 and Dhc5 from the cDNA library, under standard PCR conditions for PfuUltra II Fusion HS DNA polymerase (Catalog no. 600670, Agilent). A 6 × histidine tag encoding sequence was also added to the C-terminus of the protein sequence by PCR. *Saccharomyces cerevisiae* BJ5464-NpgA competent cells were prepared using the *S. c.* EasyCompTM Transformation Kit (Catalog no. K5050-01 Invitrogen), and transformed with the extended gene and linearized plasmid, where they underwent transformation-associated recombination (TAR). After 48 h growth on either a SDCt (A, T) or SDCt (A, U) plate, the plasmids were isolated from the cells using ZymoprepTM Yeast Plasmid Miniprep II kit (Catalog no. D2004, Zymo research). After sequence verification, the plasmids harbouring either Dhc3 or Dhc5 could be used for subsequent transformation.

Protein purification of Dhc3 and Dhc5

See Supporting Information for media recipes. Each expression plasmid harboring either Dhc3 or Dhc5 genes were transformed separately into *Saccharomyces cerevisiae* strain BJ5464-NpgA for expression. After 72 h growth on either a SDCt (A, T) or SDCt (A, U) plate, a single colony was picked and used to inoculate a 3 mL SDCt (A, T) or SDCt (A, U) seed culture, which was incubated at 28 °C and 250 rpm for 72 h. 1 mL of the seed culture was then used to inoculate a 1 L culture of YPD media and grown at 28 °C and 250 rpm for 72 h. The cells were harvested by centrifugation (5000 g, 15 minutes, 4 °C), resuspended in 25mL lysis buffer (50 mM NaH₂PO₄, pH = 8.0, 0.15 M NaCl, 10 mM imidazole) and lysed by sonication on ice (sonicate for 1 minute, then cool for 1 minute. Repeat 9 times). Cellular debris was removed by centrifugation (17000 g, 1 hour, 4 °C). Ni-NTA agarose resin (Catalog no. 30210, Qiagen) was added to the supernatant (1 mL/L of culture) and the solution was shaken at 4 °C overnight. The protein/resin mixture was loaded into a gravity flow column and proteins were purified with increasing concentration of imidazole in Buffer A (50 mM Tris-HCl, pH = 7.9, 2 mM EDTA, 2 mM DTT). Purified proteins were concentrated and exchanged into Buffer A, then concentrated and flash frozen as 20% glycerol stocks.

Isolation of DHC from double transformations

Expression plasmids harboring Dhc3 and Dhc5 genes were co-transformed into *Saccharomyces cerevisiae* strain BJ5464-NpgA for expression. After 72 h growth on a SDCt (A) plate, a single colony was picked and used to inoculate a 3 mL SDCt (A) seed culture, which was incubated at 28 °C and 250 rpm for 72 h. 1 mL of the seed culture was then used to inoculate a 1 L culture of YPD media and grown at 28 °C and 250 rpm for 72 h. 2.5mL of cyclopentanone was added after 24 h and again after 48 h to induce protein expression. Also, 25mL of 20% NaOAc was added after 24 h and again after 48 h, to increase metabolite production. The cultures were extracted with an equal volume of EtOAc, dried and concentrated *in vacuo*. The crude extract was then dissolved in 1:1 ACN:H₂O and injected onto a Luna 5_ C18(2) 100Å, AXIA, 250 × 21.2 mm reverse phase column, with detection at 202 nm. Mobile phase composition was as follows: samples were separated on a linear gradient of 20 to 95% ACN (vol/vol) in H₂O supplemented with 0.05% (vol/vol) formic

acid at a flow rate of 10 mL/min by HPLC. Initially 20% ACN, ramping up to 75% ACN over 15 min. Eluent was then increased to 95% ACN over 1.5 min, and kept there for 1 min. Eluent was then ramped down to 40% ACN over 1 min and kept there for 2 min. 11mg of DHC was typically isolated from a 1L culture.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Dehydrocurvularin gene cluster in *A. cinerariae*, and proposed biosynthetic assembly of DHC (1). KS, ketosynthase; MAT, malonyl-CoA:ACP acyltransferase; DH, dehydratase; Ψ MT, pseudo C-methyltransferase; Ψ KR, pseudo ketoreductase; ER, enoylreductase; KR_C, catalytic ketoreductase; ACP, acyl carrier protein; SAT starter-unit:ACP transacylase; PT, product template; TE, thioesterase.

Figure 2.

Phylogenetic tree of Dhc3 and other DAL or RAL producing HR PKSs. Dhc3, dehydrocurvularin (DAL); CurS1, dehydrocurvularin (DAL); Hpm8, hypothemycin (RAL); LasS1, lasiodiplodin (RAL); PKS4, zearalenone (RAL); RADS1, radicicol (RAL); ResS1, resorcylic acid (RAL). Scale bar indicates 0.05 changes per amino acid residue.

Figure 3. Surface map of AT_{Dhc3}, with mutated residues in comparison to AT_{CurS1} highlighted (beige, conserved; yellow, homologous; orange, less homologous; red, non-homologous). The majority of mutations occur on the surface and on one face of the protein. The substrate cavity, with active site serine highlighted, is found on the relatively conserved face of the protein.

Figure 4. Active site cavities of PT_{Dhc5} (cyan) and PT_{CurS2} (green, hydrophobic residues orange) with conserved tryptophan, phenylalanine, tyrosine and catalytic histidine highlighted. Substrate entrance channels are marked with an arrow.

Figure 5. Homology model of CurS2 SAT domain (green) with Dhc5 SAT domain (cyan), both generated by I-TASSER^[36–38] with no external structural restraints added. The two homology models overlay extremely well.

Table 1

Domain comparison of Dhc3 and Dhc5, with CurS1 and CurS2, respectively. The core of Dhc3 consists of ψ MT and ψ KR subdomains.

Protein	Domain	% Sequence Identity (homology)
Dhc3	KS	87 (94)
	AT	78 (87)
	DH	77 (86)
	core	52 (70)
	ER	86 (91)
	KR	83 (91)
	ACP	83 (86)
Dhc5	SAT	71 (83)
	KS	93 (96)
	AT	76 (88)
	PT	78 (89)
	ACP	83 (93)
	TE	85 (90)