Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 22, No. (1) : 2020 : 120-132

ISSN-0972-3005

THE *DICTYOSTELIUM DISCOIDEUM* DIPKS14: A MEMBRANE-ANCHORED HIGHLY REDUCING ITERATIVE POLYKETIDE SYNTHASE SIMILAR TO ONES FOUND IN PATHOGENIC FUNGI AND MYCOBACTERIA

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(Received 31 October, 2019; accepted 24 November, 2019)

***Key words**: Polyketide synthase, Dictyostelium discoideum, Phylogeny, Molecular modeling*

Abstract – Naturally occurring iterative type I polyketide synthase enzymes are known to make therapeutic polyketide molecules that act against bacteria, cancer and cholesterol. Identifying and characterizing these enzymes will help develop novel therapeutics. The model organism *Dictyostelium discoideum* is a soil-dwelling social amoeba, one of the largest known repositories of type I polyketide synthases and is a potential host system for metabolite and biologics production. Of the 45 *Dictyostelium discoideum* polyketide synthases (PKS) 39 are Type-I PKSs and none has been characterised. PKSs are condensing enzymes that string together two carbon units to generate a polyketide lipid chain. Type I highly reducing polyketide synthases are characterized by the presence of multiple catalytic domains on the same polypeptide. These domains are used to first join the subunits and then variously and iteratively reduce, dehydrate, further reduce and finally release the polyketide chain. Here we use bioinformatics and homology based structural modeling to characterize the *Dictyostelium discoideum* polyketide synthase 14 (DiPKS14). DiPKS14 is a polyketide synthase with evolutionary connections to PKSs from pathogenic fungi and bacteria. Multiple domains on the same polypeptide identify it as a type I PKS enzyme whereas the presence of several reducing catalytic domains define it as a highly reducing type I PKS enzyme. The presence of a C-terminal extended reductase domain confirms that it is a chain releasing enzyme. A polar- non-polar di-domain precedes the conserved catalytic triad of the reductase domain. Two transmembrane regions, one in the enoyl reductase domain and the other in the extended reductse domain predict a membrane anchored PKS.

INTRODUCTION

Polyketide synthases produce a diverse range of lipid metabolites that are useful as medicines and contribute to the structure and function of cell membranes, cell and spore walls in plants and bacteria (Cane, 1998; Gokhale and Tuteja, 2001; Kim *et al.,* 2010; Porter *et al.,* 2013; Anand *et al.,* 2015). Identifying and characterizing these enzymes from new organisms will contribute to the knowledge base of therapeutics. Type I Polyketide synthases are multifunctional enzymes similar to fatty acid synthases and have multiple catalytic domains on the same polypeptide (Hutchinson *et al.,* 2000). The difference between the two lies in the ability of polyketide synthases to use small chain and long chain substrates apart from acetyl CoA and malonyl CoA to assemble the longer chain lipids to make polyketides (Anand *et al.,* 2015). Whereas fatty acid synthases use all the catalytic domains in the enzyme in a fixed order, polyketide synthases can use the catalytic domains in various combinations during each iteration to produce a diverse range of metabolites (Halo *et al.,* 2008; Ma *et al.,* 2009). The catalytic domains ketosynthase, acyltransfease, dehydratase, enoyl reductase, ketoreductase, acyl carrier protein are present in the same order as in FASs, however certain type I PKS such as the lovastatin nonaketide synthase also have a C-methyl transferase domain and an extended C-terminal reductase domain that is similar to the condensation domains of non-ribosomal peptide synthases (Hutchinson *et al.,* 2000). *Mycobacterial* iterative PKSs (iPKS) that make cell envelope components also have extended reductase domains. These are known as highly reducing iterative type I polyketide synthases also commonly found in filamentous fungi (Cox, 2007). Highly reducing

iterative type I polyketide synthases produce polyketides in which the ketone groups are highly reduced by the combined action of the ketoreductase, dehydratase and enoyl reductase domain (Halo *et al.,* 2008). Iterative Type I PKS are commonly found in pathogenic fungi and make both aromatic compounds such as 6-methylsalicylic acid and the spore pigment precursor wA and non-aromatic compounds such as brefeldin A, monacolin K, and T-toxin. Type I polyketide synthases also make polyene lipids with alternating double and single bonds such as linearmycins (Hoefler *et al.,* 2017) and fumonisin B in which oxygen atoms from molecular oxygen and methyl groups from SAM-dependent methyl transferases contribute to the lipid chain. Sequence based domain analysis has been useful in large scale predictions of polyketide synthase substrate specificity and catalytic functions (Yadav *et al.,* 2009). In this study we use bioinformatics and homology based structural modeling techniques to identify and characterize the *Dictyostelium discoideum* polyketide synthase 14 or DiPKS14 as a membrane anchored iterative highly reducing type I polyketide synthase with a C-terminal reductase domain and present a schematic model representing its structure.

MATERIALS AND METHODS

DNA Sequences were obtained from dictyBase (Gaudet *et al.,* 2008) and NCBI (Brown *et al.,* 2015). Exon and intron regions were annotated based on open reading frames and sequence homology to other polyketide synthase genes. Domain boundaries within the protein were identified by conserved domain analysis performed on the protein sequence at the conserved domain database at NCBI (Marchler-Bauer *et al.,* 2017). Phylogenetic analyses were conducted using the entire sequence or just the ketosynthase domain using the BLAST tool (Altschul *et al.,* 1990) with local sequence alignment allowing for gaps. BLAST results were then used for building phylogenetic trees using distance tree formation by neighbor joining. Sequences corresponding to the conserved domains were then aligned by neighbor joining using the Clustal Omega tool at EBI (Sievers *et al.,* 2011). Conserved motifs corresponding to each domain were identified by the consensus sequences obtained from multiple sequence alignment and verified by bio-curation. Both homology searches and conserved domain searches were assumed to be valid if the query cover was more than 60%, the p-value was less than 1.0e-03 and the percent identity was greater than 25%. Distance matrices were obtained by pairwise alignment of all BLAST hits for the full length DiPKS14 protein sequence using the Clustal Omega tool. Homology based structural modeling was conducted at Swiss-Model (Waterhouse *et al.,* 2018) using two templates with the highest homology from fifty possible templates.

RESULTS

Identifying the *dipks14* gene

The *dipks14* gene was identified by a search at the Dictyostelium database dictyBase for the term polyketide synthase. The search term returned a list of 45 putative polyketide synthase genes (*dipks*) of which *dipks14* and *dipks14* had already been identified and characterized. Further analysis revealed the presence of two conserved full length polyketide synthase genes *dipks14* and *dipks18* that had 68% identity and 81% similarity and 3% gaps with each other and a minimum of 45% similarity with the other *Dictyostelium discoideum* polyketide synthases. The DiPKS14 gene is present on two exons on chromosome 2 of the *Dictyostelium discoideum* genome (Figure 1). Exon one contains the first 661bp of the coding region with the chromosomal co-ordinate 2081242 to 2080582 and encodes half of the N terminal ketosynthase domain. Exon2 has 8335bp and is contained in the chromosomal co-ordinates 2080478 – 2072143 and encodes the rest of the protein.

The domain architecture of DiPKS14

The domain architecture of the DiPKS14 protein was computationally analysed (Figure 2). The 2998 amino acid protein sequence was retrieved from dictyBase and subject to a conserved domain analysis at the CDD database at NCBI. Conserved domain analysis identified eight conserved domains on a single polypeptide. The eight conserved domains contained the core domains ketosynthase (KS), acyltransferase (AT), ketoreductase (KR) and acyl carrier protein (ACP) (Table 1). In addition there were four more domains dehydratase (DH), C-methyltransferase (C-Met), enoyl reductase (ER) and a C-terminal extended short chain dehydrogenase domain. The presence of these eight domains on a single polypeptide along with multiple reducing

domains identified the DiPKS14 gene as a highly reducing type I PKS enzyme with a sequential arrangement of the domains as follows. KS (20-454, yellow), AT (576-872, blue), DH (965-1244, purple), C-Met (1448-1564, green), KR (1858-2166, grey), ER (2208-2388, orange), ACP (2456-2573, teal), R (2620-2936, pink) (Figure 2).

Phylogenetic analysis of the DiPKS14 enzyme

Phylogenetic analysis of the DiPKS14 enzyme was conducted with the sequences of just the KS domain or the entire protien sequence. The two analyses gave different but overlapping results. Phylogenetic analysis with the sequence representing the ketosynthase domain alone gave a larger number of hits that included polyketide synthases from other dictyostelids such as *Dictyostelium purpureum*, *Dictyostelium fasciculatum* and *Heterostelium album* (Figure 3). This list also included polyketide synthases from bacterial polyketide synthases. Phylogenetic analysis with the entire DiPKS14 protein sequence gave a list of homologous proteins that represented polyketide synthases from the dictyostelid members and a few fungal polyketide synthases (Figure 4).

The phylogenetic analysis of the N-terminal ketosynthase domain of the DiPKS14 protein revealed a close association of polyketide synthases in the descending order with the polyketide synthases from *Dictyostelium discoideum*, followed by PKS from other dictyostelids, followed by bacterial and fungal polyketide synthases. DiPKS14 was most similar to DiPKS18 ((Figure 4) red boxes, greater than 80% identity), it was approximately 50%

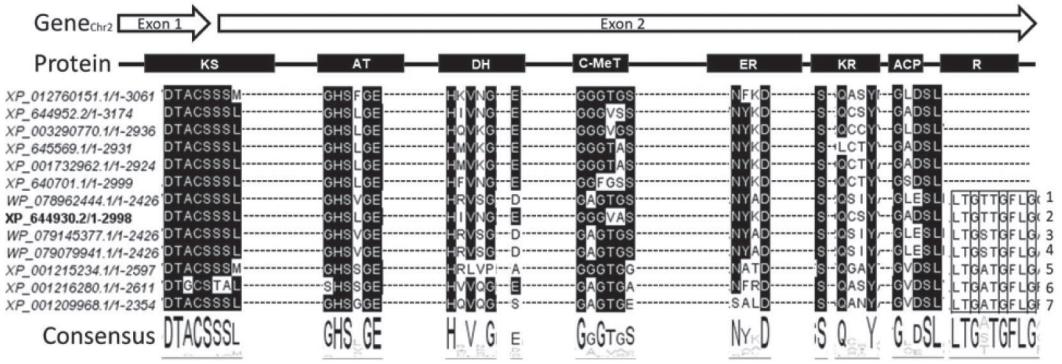

Fig. 1. Schematic of the DiPKS14 gene organization, protein domain architecture, and conserved motifs. Exons (white arrows), catalytic domains (black boxes), conserved motifs (multiple sequence alignment), motif consensus (last row). Exon 1 is 662bp in length and Exon 2 is 8335bp in length. Catalytic domains KS ketosynthase, AT acyltransferase, DH dehydratase, C-MeT C-methyltransferase, ER enoyl reductase, KR ketoreductase, ACP acyl carrier protein, R reductase. The R domain is aligned with the reductase domains of 1. DiPKS6, 2. DiPKS9, 3. DiPKS14, 4.DiPKS18, 5. 4U7W MyxA *Myxobacteria* NRPS, 6.4U5Q *Mycobacterium tuberculosis* NRPS, 7. 4DQV *Mycobacterium tuberculosis* NRPS. XP_644930.2 is DiPKS14 (bold), XP_012760151.1 is *Acytostelium subglobosum* hypothetical protein, XP_644952.2 is DiPKS15, XP_003290770.1 is *Dictyostelium purpureum*hypothetical protein, XP_645569.1 is DiPKS9, XP_001732962.1 is DiPKS6, XP_64070.1 is DiPKS18, WP_078962444.1 is *Streptomyces hygroscopicus* PksD, WP_079145377.1 is *Streptomyces hygroscopicus*PksD, WP-079079941 is *Streptomyces hygroscopicus* PksD, XP_00125234.1 is *Aspergillus terreus* Pks, XP_001216280 is *Aspergillus terreus* Pks, XP_001209968 is *Aspergillus terreus* Pks.

Table 1. Conserved domains detected on the DiPKS14 protein sequence.

List of domain hits DiPKS14				
Name	Accession	Description	Interval	E-Value
KS	smart00825	Polyketide synthase beta-ketoacyl synthase	22-456	2.35E-115
AT	smart00827	Polyketide synthase acyltransferase	576-872	1.96E-84
DH	pfam14765	Polyketide synthase dehydratase	965-1244	6.60E-09
C-MeT	pfam08242	Methyltransferase	1451-1564	7.16E-12
ER	cd05195	Polyketide synthase enoyl reductase	1858-2166	1.21E-79
KR	pfam08659	Polyketide synthase ketoreductase	2208-2388	9.71E-17
ACP	N/A	Acyl carrier protein	2456-2473	MI
R	COG3320	NRPS reductase domain	2620-2881	1.42E-29

identical to the *Dictyostelium discoideum* polyketide synthases DiPKS6, DiPKS9, DiPKS15 and DiPKS45. The DiPKS14 protein was more identical to the Ascomycete Fungi *Aspergillus terreus* polyketide synthases (30% identical) and least identical to the

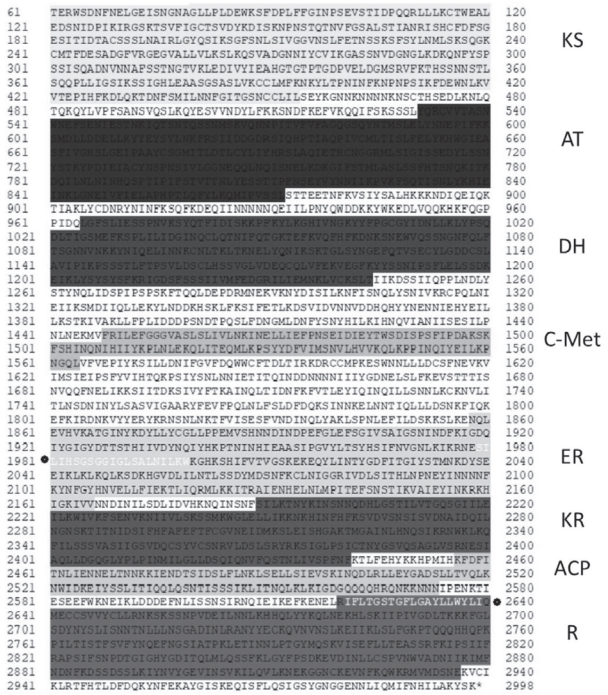

Fig. 2. Sequence and domains of DiPKS14. The 2998 amino acid sequence of DiPKS14 has eight conserved domains the KS (20-454, yellow), AT (576-872, blue), DH (965-1244, purple), C-Met (1448- 1564, green), KR (1858-2166, grey), ER (2208-2388, red), ACP (2456-2573, teal), R (2620-2936, pink).
Two membrane anchoring domains (white lettering) are present in the ER and R domains

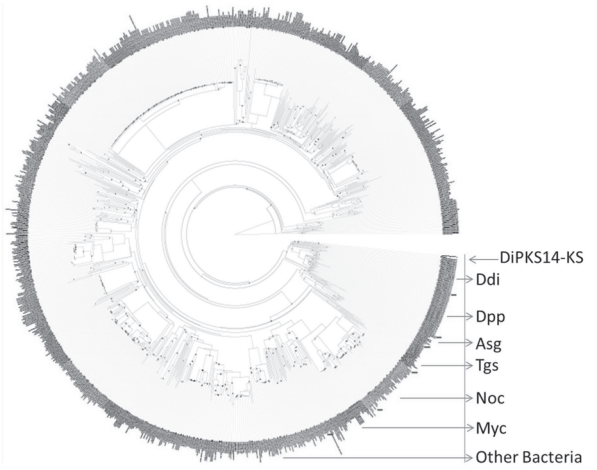

Fig. 3. Phylogenetic analysis of the N-terminal protein sequence of the ketosynthase domain of DiPKS14. Circular phylogenetic tree of the evolutionary relationship of the N-terminal ketosynthase domain of DiPKS14 with that of polyketide synthases from other dictyostelids and bacteria. Dpp *Dictyostelium purpureum*, Asg: *Acytostelium subglobosum*, Tgs: *Tieghemostelium lacteum*, Noc: *Nocardia spp.*, Myc: *Micobacterium spp.*

Actinomycete bacteria *Streptomyces hygrosopicus* polyketide synthases (less than 21% identical).

Identification of the conserved motifs in each domain

The identity of each of the catalytic domains of the DiPKS14 enzyme was further validated by identifying the conserved motifs within the domain by a multiple sequence alignment with other *Dictyostelium discoideum* polyketide synthases and PKS from fungi which possess many type I highly

DiPKS14 %ID Matrix		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	
1	ABI93779.1	100	31.6	31.27	31.27	24.55	25.51	25.3	23.31	22.26	22.95	19.19	19.89	20.81	20.42	20.27	19.83	20.05	
2	WP_078962444.1	31.6	100	98.47	98.35	28.56	31.39	29.98	26.85	23.68	26.03	22.06	22.07	22.83	21.87	21.71	21.99	21.75	*S.hygroscopicus*
3	WP_079145377.1	31.27	98.47	100	99.71	28.12	31.13	29.88	26.67	23.55	25.89	21.83	21.85	22.7	21.69	21.53	21.94	21.53	
4	WP_079079941.1	31.27	98.35	99.71	100	28.08	31.09	29.88	26.67	23.59	25.89	21.83	21.81	22.7	21.64	21.48	21.94	21.53	
5	XP_001215234.1	24.55	28.56	28.12	28.08	100	31.35	29.78	24.55	22.98	23.11	20.52	20.73	21.35	21.56	21.64	20.7	20.99	
6	XP_001216280.1	25.51	31.39	31.13	31.09	31.35	100	35.93	25.26	23.69	24.8	21.4	21.54	21.87	21.74	21.52	21.36	21.36	
7	XP_001209968.1	25.3	29.98	29.88	29.88	29.78	35.93	100	25.19	24.69	25.14	23.55	21.89	22.3	22.48	22.18	22.82	22.16	*A.terreus*
8	XP_012760151.1	23.31	26.85	26.67	26.67	24.55	25.26	25.19	100	35.82	36.75	29.07	30.1	30.48	30.74	30.55	31.6	30.79	
9	XP_020435993.1	22.26	23.68	23.55	23.59	22.98	23.69	24.69	35.82	100	60.14	32.44	34.81	35.34	34.36	34.41	36.15	36.65	
10	XP_004366063.1	22.95	26.03	25.89	25.89	23.11	24.8	25.14	36.75	60.14	100	33.26	35.17	35.3	34.07	34.49	35.72	36.06	
11	Q54B49.2	19.19	22.06	21.83	21.83	20.52	21.4	23.55	29.07	32.44	33.26	100	38.19	39.69	39.56	39.34	40.27	39.68	
12	XP_644952.2	19.89	22.07	21.85	21.81	20.73	21.54	21.89	30.1	34.81	35.17	38.19	100	48.64	51.78	51.12	58.4	55.76	
13	XP_003290770.1	20.81	22.83	22.7	22.7	21.35	21.87	22.3	30.48	35.34	35.3	39.69	48.64	100	49.51	49.56	50.14	49.23	
14	XP_645569.1	20.42	21.87	21.69	21.64	21.56	21.74	22.48	30.74	34.36	34.07	39.56	51.78	49.51	100	92.98	54.69	52.95	*D.discoideum*
15	XP_001732962.1	20.27	21.71	21.53	21.48	21.64	21.52	22.18	30.55	34.41	34.49	39.34	51.12	49.56	92.98	100	54.61	53.09	
16	**XP_644930.2**	**19.83**	**21.99**	**21.94**	**21.94**	**20.7**	**21.36**	**22.82**	**31.6**	**36.15**	**35.72**	**40.27**	**58.4**	**50.14**	**54.69**	**54.61**	**100**	**69.93**	
17	XP_640701.1	20.05	21.75	21.53	21.53	20.99	21.36	22.16	30.79	36.65	36.06	39.68	55.76	49.23	52.95	53.09	69.93	100	

S.hygroscopicus *A.terreus* *D.discoideum*

Fig. 4. Percent Identity Matrix of the protein sequence DiPKS14 with other DiPKS and fungal PKSs. The percent ID matrix of DiPKS14 with other polyketide synthases from *Dictyostelium discoideum* (*D.discoideum*), *Aspergillus terreus* (*A.terreus*) and *Streptomyces hygroscopicus* (*S.hygroscopicus*). Red indicates highest homology and blue indicates less homology.

reducing polyketide synthases, that of *Aspergillus terreus* and *Streptomyces hygroscopicus* (Figure 1) and the proteobacteria *Myxobacterium*.

The ketosynthase domain

The ketosynthase catalytic domain of polyketide synthases is a condensing enzyme that catalyzes the claisen condensation between a small chain extender unit such as an acetyl CoA, malonyl CoA or methyl malonyl CoA unit from the cellular pool and the growing polyketone chain tethered to the enzyme. Conserved domain analysis reveals that the DiPKS14 ketosynthase domain extends from amino acids 0-445. Using the compositional matrix adjust program at the NCBI BLAST two sequences tool, we were able to show that ketosynthase domain of DiPKS14 was 53% similar (243aa/458aa), and had an identity of 34% (158aa/458aa) with the ketosynthase domain of the lovastatin nonaketide synthase a highly reducing type I fungal Polyketide synthase. A gap of 6% (31aa/ 458aa) was observed (Table 2).

Multiple sequence alignment with other ketosynthase domains from PKS genes from *Dictyostelium discoideum*, *Aspergillus spp.*, *Streptomyces spp.*, along with the Lovastatin biosynthetic PKS, showed that DiPKS14 has all the conserved motifs (Figure 5) present in ketosynthase domains of polyketide synthases (Bhetariya, 2016). The ketosynthase domain was identified by the presence of the conserved serine S190 residue within the conserved motif D186TACSSSL193 and the catalytic triad of C189, H331 and H376 of the ketosynthase domains of fungal type I PKS's that are also conserved in DiPKS14.

The acyltransferase domain

The acyl transferase domain loads the starter unit (which can be a specific type of acyl-CoA) to the acyl carrier protein this allows for the extension of the chain by the ketosynthase domain that makes a C-C bond between the growing chain attached at the KS and the starter unit (Gokhale *et al.*, 1998). To

Table 2. Homology of the DiPKS14 and the Lovastatin highly reducing type I polyketide synthase ketosynthase domains.

Method	Queryaccession	Query Description	Score	e-value	Query cover	Identities	Positives	Gaps
Compositional matrix	lcl | Query _244407	AAD39830.1 lovastatin adjust. nonaketide synthase [*Aspergillus terreus*]	361bits (926)	3e-103	50%	281/1037 (27%)	481/1037 (46%)	85/1037 (8%)

Method is the method used for BLASTing two protein sequences. Query accession is the NCBI ID of the sequence against which the DiPKS14 ketosynthase domain was blasted. Score represents the quality of the alignment with higher scores representing higher quality. E-value, represents the probability of finding a similar alignment. Query cover represents the length of the alignment. Identities represent the percent of identical residues. Positives represent the number of similar residues. Gaps represent mismatches and an intermediary gap penalty is ideal.

Table 3. Q-Mean and GMQE values for the models of the DiPKS14 reductase domain.

Model	Gene / Genus	Sequence Identity	Sequence Similarity	Q Mean	GMQE	Coverage
4u7w.1	MxaA /*S. aurantiaca*/ Myxo Bacterium	25.07%	0.32	-2.93	0.61	0.91
4dqv.1.A	Nrp/*M. tuberculosis*/ Mycobacteria	19.51%	0.29	-4.38	0.59	0.96
4u5q.2.A	Peptide Synthatase	19.51%	0.29	-4.57	0.58	0.96

Model gives the PDB ID of the protein domain. Gene and genus is the gene name and species name of the source organism. Sequence identity is the percent of identical residues. Sequence similarity is the percent of residues with similar properties. Q Mean defines the quality of the protein with scores of -4 and above being good quality. GMQE scores represent reliability with scores between zero and one, with the higher score being more reliable. Coverage defines the alignment length with higher numbers giving better coverage.

determine the conservation of the acyltransferase domain of the DiPKS14 enzyme we conducted MSA of the DIPKS14 acyltransferase domain (576-872) with other *Dictyostelium discoideum* type I PKS and those from *S.hygroscopicus* and *A.terreus* (Figure 1).

We confirmed the presence of the conserved serine S667 within the conserved G665HSXGE670 motif (Figure 1) present in the acyltransferase AT domain (Khayatt *et al.,* 2013; Gay *et al.,* 2014; Sychantha *et al.,* 2018) of DiPKS14. AT's contain an invariable glutamine (Q583 in DiPKS14) at their catalytic site along with a histidine and serine. A second conserved motif Q583XXXMXXXL591 was also present.

The dehydratase domain

Dehydratase domains remove a hydroxyl group to form a double bond in the β position of the newly added two carbon extender unit of the growing Polyketide chain. Dehydratase domains have a hot-dog 3D structure and have an invariant and conserved aspartate and histidine amino acid in the catalytic active site (Akey *et al.,* 2010). The DiPKS 14 dehydratase shows 30-61% identity to other dehydrates domains in pairwise alignments. Two invariant and conserved aspartate E1028 and histidine H996 amino acid which are present in the dehydratase catalytic active site of DiPKS14 are also

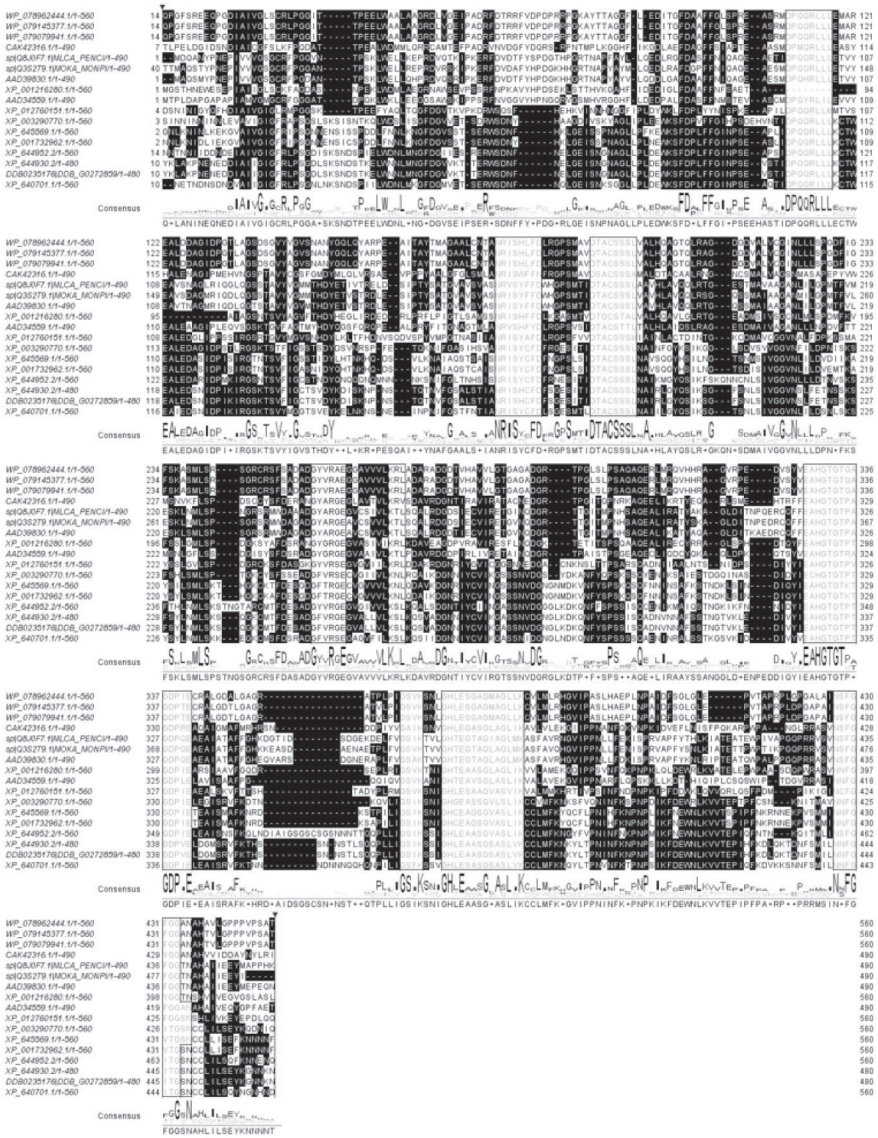

Fig. 5. Multiple sequence alignment of the ketosynthase domain of DiPKS14. Boxes with light lettering indicate conserved motifs. Amino acids highlighted in black are conserved. The consensus line indicates the residues present most often at that position across species. The species name and gene description are as in Figure 1.

conserved (Figure 1).

The enoyl reductase domain

Enoyl reductase reduces the double bond formed by the dehydratase domain to saturate the β carbon of the newly incorporated C2 unit. The ER catalytic domain is composed of two domains a core cofactor (NADPH)-binding domain and a discontinuous substrate binding or catalytic domain (Ames *et al.*, 2012).

Enoyl reductases are conserved domains with a substrate binding site with the consensus NFRD(hy)4G with four hydrophobic residues (hy)4. The DiPKS14 ER domain has the following conserved domain at its N terminus. N1871YKD(YLLY)CG1879 (Figure 1). T1849 and T1208 are both completely conserved. We did not find the canonical GXXGXXG NADP binding Rossmann fold consensus sequence however a glycine rich motif between residues **G**1898LEFS**G**IVSAI**G**SNINDFKI**G**DQIY**G**I**G**1925 has several well conserved glycines (Figure 6).

The C-methyl transferase domain

The methyl transferase domains add a methyl group to the carbon atom of the newly added unit using an S-adenosyl methionine or SAM donor. There are three motifs in the methyltransferase domain that are highly conserved among all methyltransferases, as they facilitate SAM binding to the structure. These are the G-loop (motif I), the D-loop (motif II), and the P-loop (motif IV) motifs (Ansari *et al.*, 2008). The G-loop interacts with the amino acid portion of SAM. The D-loop contains an acidic residue, either Aspartate or Glutamate, whose side-chain hydrogen bonds with the ribose hydroxyl of SAM. The P-loop, located at the C-terminal end, is a proline-rich motif that binds with the adenine ring of SAM via hydrophobic interactions. The identity of the C-methyl transferase domain was confirmed by the presence of three conserved domains, an N terminal glycine-rich motif G1454GG1456, the following conserved domain at its N terminus N1871YKD(YLLY)CG1879 (Figure 1), and two conserved acidic residues Glutamate E1452 and Aspartate D1488 and two conserved proline residues P1549 and P1550

The ketoreductase domain

The DiPKS14 ketoreductase domain from (2189-

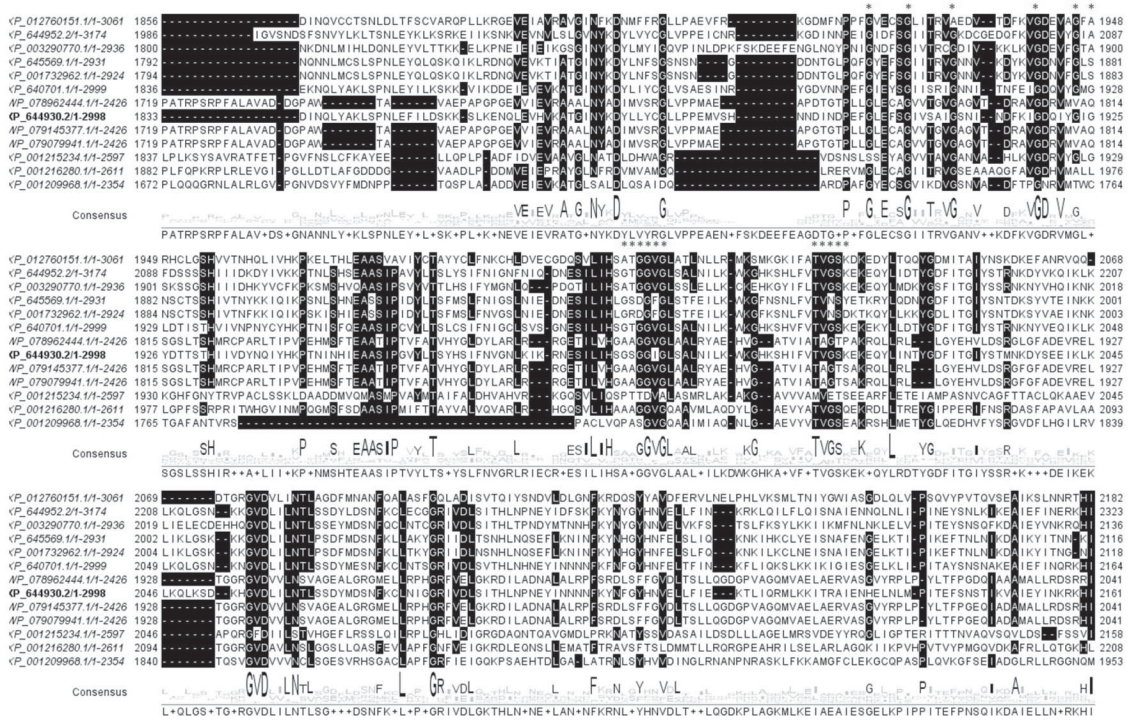

Fig. 6. Multiple sequence alignment of the enoyl reductase domain of DiPKS14. Conserved domains are highlighted in black, important residues are marked in red asterix. Gaps are indicated as dotted lines and the consensus line indicates the amino acid with the highest probability of being in that position. Accession number and the corresponding protein and organism can be obtained from the caption of Figure 1.

2441) was confirmed by the presence of two conserved amino acids tyrosine Y2359 and serine S2346 (Figure 1). The ketoreductase domain catalyzes the reduction of the keto group of the freshly added C2 two carbon extender to a hydroxyl group. This conversion is done by the conserved serine and tyrosine residues at the catalytic site (Xie *et al.*, 2016).

The ACP domain

The acyl carrier protein (ACP) domain is one of the core domains of the polyketide synthase enzyme. The modification of the ACP domain involves the transfer of a 340Da phosphopantetheine group derived from coenzymeA (CoA) on to the hydroxyl group of a serine residue in the ACP domain (Charkoudian *et al.*, 2011). The DiPKS14 ACP domain was manually annotated since it was not revealed in the search at the Conserved Domain Database at NCBI. In a search for a conserved serine residue, the ACP domain was identified as existing between the ketoreductase and extended reductase domain. DiPKS14 has a conserved serine residue S2513 within the conserved motif G2510XXSL2514, within the ACP domain (Figure 1).

The reductase domain

The C-terminal domain of DiPKS14 represents an extended short chain dehydrogenase. This region did not align with the C-terminal domains of fungal type I PKSs such as the ones that showed highest homology to DiPKS14. Instead a separate multiple sequence alignment was conducted with the reductase domains of *Dictyostelium discoideum* DiPKS6, DiPKS9, DiPKS15 and the reductase domains of *Mycobacteria* nonribosomal peptide synthases 4U5Q and 4DQV and the *Myxobacterial* reductase domain 4U7W from a MyxA NRPS. The conserved canonical GXXGXXG NADP binding domain is present in the DiPKS14 reductase domain as TG2625STG2628FLG2631 (Figure 1) as are the catalytic triad T2767, Y2795 and K2799 (Figure 7). Two hydrophobic conserved residues, tryptophan W2927, and valine V2931 were also identified. In addition just upstream of the region containing the catalytic triad the presence of a string of polar residues Q2753QQHHQ2758 (Figure 7) is a novel feature in the DiPKS14 reductase domain, not seen in the other sequences analysed in this study.

The DiPKS14 predicted R domain sequence was modeled at SwissProt (Figure 9) and a model was built successfully using the templates of monomeric R domains of three peptide synthetases 4u7w.1, 4dqv.1.A and 4u5q.2. A with a sequence homology of 32%, 29% and 29% respectively. Modeling could not be done on the thioesterase proteins 5msp.1.A

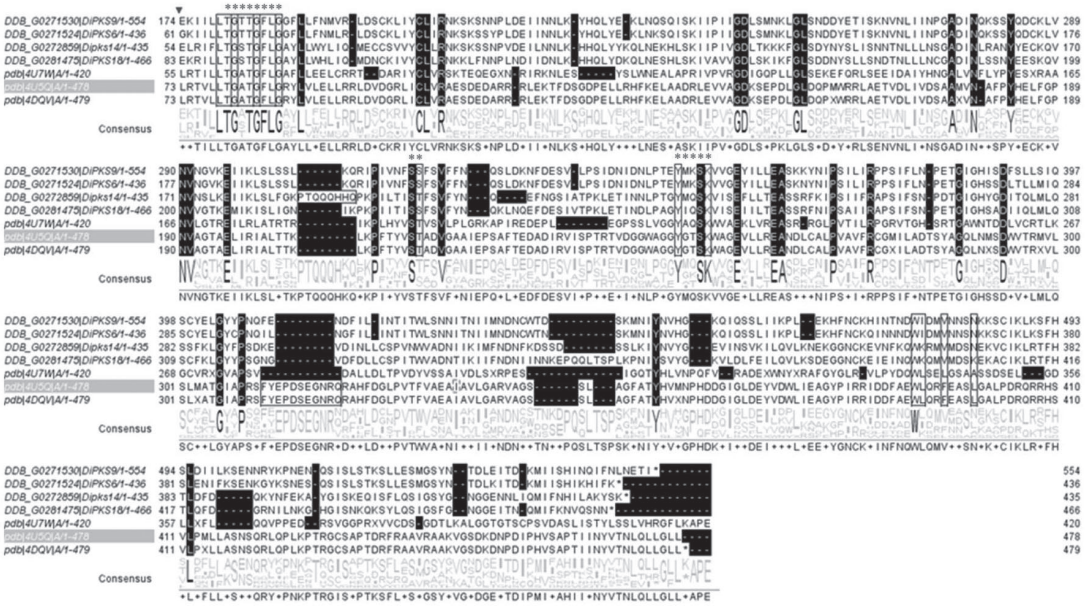

Fig. 7. Multiple sequence alignment of the C-terminal reductase domain of DiPKS14. Conserved residues are highlighted in black. Conserved motifs are boxed in black. The consensus line indicates the amino acid with the highest probability of being in that position. Accession number and the corresponding protein and organism can be obtained from the caption of Figure 1.

and 5msv.1.A and the 5msv.1. A carboxylic acid reductases although they had nearly as high a homology as the peptide synthetases. We show a computationally derived 3D structural model of the DiPKS14 reductase domain based on the available structures of 4u7w (Figure 8) and 4dqv which are reductase domains from *Mycobacterial* and *Myxobacterial* nonribosomal peptide synthases.

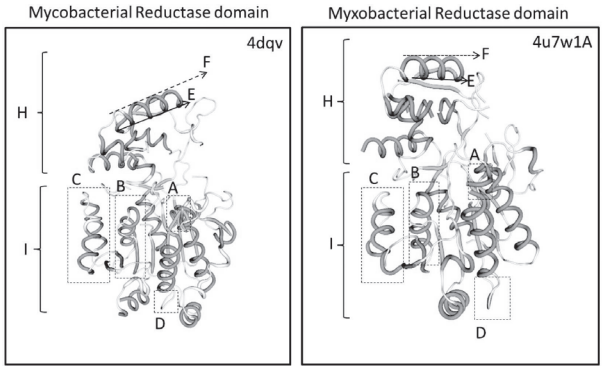

Fig. 8. Comparative tube diagrams of reductase domains. Reductase domain 4U7W MyxA *Myxobacteria* NRPS and 4DQV *Mycobacterial* NRPS Reductase Domain. Boxes: A- catalytic triad, B- a representative Rossmann fold α/β structure, C-helix turn helix, D-unstructured polar domain, F-hydrophilic side of helix, E - hydrophobic side of helix, H- C terminal sub domain (substrate binding), I- N-terminal subdomain (NADPH binding).

Conserved domain analysis has shown the presence of an extended C-terminal reductase short chain dehydrogenase domain in DiPKS14. Polyketide synthases (PKS) and nonribosomal peptides (NRPS) from *Mycobacterium tuberculosis* and *Stigmatella aurantiaca* a *Myxobacteria* possess a C-terminal extended NADP dependent off-loading reductase domain (Chhabra *et al.*, 2012; Barajas *et al.*, 2015). This off-loading domain releases the thioester bound lipids and lipopeptides by a reductive reaction. These reductase domains have classic GXXGXXG glycine rich NADP binding domains, a catalytic triad of threonine (T), tyrosine (Y) and lysine (K) and the hydrophobic residues tryptophan (W), leucine (L), phenyl alanine (F), leucine (L), that form the hydrophobic pocket of the substrate binding site. To confirm the presence of an R domain in DiPKS14 we conducted multiple sequence alignments of DiPKS14 with other reductase domain containing *Dictyostelium discoideum* PKS protein coding sequences with that of a few off -loading reductases from *Mycobacteria* and *Myxobacteria* for which the structures have been solved.

The structure of the DiPKS14 reductase domain consists of two domains (Figure 9). Firstly, an N terminal domain (I) with the extended NADPH binding with a α/β Rossmann fold with beta strands (Figure 9 green) flanked by alpha helices (blue). One of the α/β Rossmann fold structures are highlighted as (B) with the alphas helix in blue alternating with the beta strand in green. The Rossmann fold is a characteristic feature of the SDR family of oxidoreductases that have a conserved tyrosine residue in the catalytic domain TG2625STG2628FLG2631 that comprises the classic signature of GXXGXXG conserved glycine rich NADPH binding Rossmann fold motif. Multiple sequence alignment and structure based alignment both confirm the presence of the catalytic triad (A) in DiPKS14 reductase domain T2767 (QMEAN 0.69), Y2795 (QMEAN 0.67) and K2799 (QMEAN 0.73), are present at similar positions as seen in other similar proteins (Table III). Present also is the helix-turn-helix that is characteristic of the C-terminal extended reductase domain in PKS/NRPS systems (C). The DiPKS14 reductase domain has an extra feature of a short polar domain in N terminal domain that consists of highly polar residues Q2753QQHHQ2758 (D). This region is absent in the *Mycobacterial* and *Myxobacterial* C-terminal reductase domains.

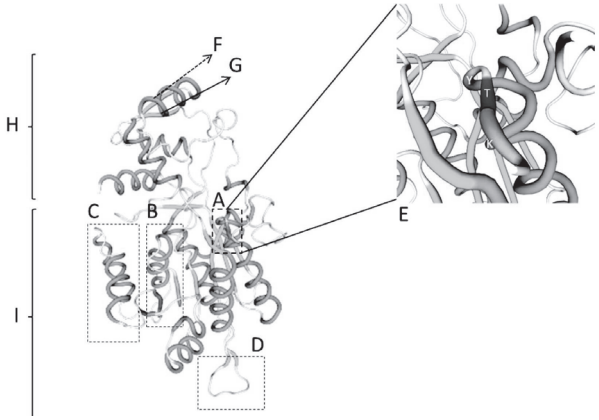

Fig. 9. Comparative tube diagrams of reductase domains. Model of the reductase domain of DiPKS14. Boxes: A- catalytic triad, B- a representative Rossmann fold α/β structure, C-helix turn helix, D-unstructured polar domain, E- Catalytic triad residues, F- hydrophilic side of helix, G-hydrophobic side of helix, H- C terminal sub domain (substrate binding), I- N-terminal subdomain (NADPH binding).

Secondly, a C terminal domain (Figure 9) also called a substrate binding domain (H) is present in the reductase domain. This is characterized by a helix with an inner hydrophobic side (G) and an outer hydrophilic side (F).

A. The two transmembrane regions and a non-polar stretch in DiPKS14

The DiPKS14 protein sequence analysis revealed two trans-membrane domains that are each approximately 20 amino acids long (Figure 10). The first is present in the enoyl reductase domain and extends from serine S1979 to tryptophan W1999 (S1979ILIHSGSGGIGLSALNILKW1999). The second transmembrane domain is at the N-terminus of the extended reductase domain and extends from isoleucine I2619 to isoleucine I2639 (I2619FLTGSTGFLGAYLLWYL$_I$2639).A polar domain not present in other closely related *Dictyostelium discoideum* PKS proteins extends from Q2753 to Q2758 and is succeeded by a highly non-polar region P2761ILTISTFSVFY2772.

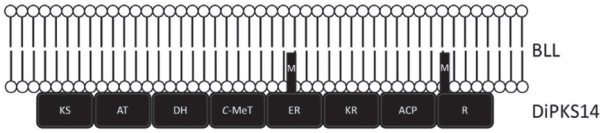

Fig. 10. Schematic representation of the domain architecture of DiPKS14 enzyme. Catalytic domains are represented by black boxes with labels. Ketosynthase (KS), acyltransferase (AT), dehydratase (DH), C-methyltransferase (C-MeT), enoyl reductase (ER), ketoreductase (KR), acyl carrier protein (ACP), extended reductase (R) . Black bars represent membrane anchors in the ER and R domains. Bi lipid layer (BLL), *Dictyostelium discoideum* polyketide synthase 14 (DiPKS14).

DISCUSSION

Dictyostelium discoideum has a large number of secondary metabolic enzymes including the nonribosomal peptide synthases and the polyketide synthases. There are 45 predicted polyketide synthases (Eichinger *et al.,* 2005; Ghosh *et al.,* 2008). Dictyostelium is known to make several natural products (Takaya *et al.,* 2000, 2001; Kikuchi, 2007; Kubohara *et al.,* 2017) including the polyketide synthase products DIF1 and MPBD (Austin *et al.,* 2006; Ghosh *et al.,* 2008; Manocha, 2011) and is a natural source of therapeutic molecules. Characterizing these enzymes in silico has been essential to their identification and isolation and subsequent cellular and biochemical characterization. This is especially true for megasynthases like the polyketide synthases where often domain identification by bioinformatics techniques has been indispensable for downstream biochemical characterization of the individual catalytic domains. Here we use bioinformatics and homology based structural modeling to identify and characterize the *Dictyostelium discoideum* polyketide synthase 14. Phylogenetic tree analysis verified that DiPKS14 is a polyketide synthase with homology to dictyostelid, fungal and bacterial polyketide synthases. Identi- fication of all eight catalytic domains including a C-methyltransferase domain and an extended reductase domain on a single polypeptide defined it as a type I polyketide synthase of the secondary metabolism.

The presence of extra domains such as the enoyl reductase and the dehydratase specify that it is a highly reducing type I polyketide synthase. Homology based structural modeling confirms that the C-terminal extended short chain dehydrogenase domain is similar to the reductase domains of bacterial non-ribosomal peptide synthases that release polyketide products as alcohols and aldehydes rather than acids as would be the case in thioesterase domains. The reductase domain has a six residue long polar region just upstream of the catalytic triad and this region is followed by a 12 residue long non-polar domain which we identify as the polar-nonpolar bi-domain. Conserved domain analysis revealed two "gap" regions in the enoylreductase and reductase domains. On closer observation the gaps represented approximately twenty amino acid long hydrophobic patches. The N terminal region of the reductase domain has a string of 19 hydrophobic amino acids and the enoyl reductase domain has a central 21aa hydrophobic region both predicted to be a transmembrane domains. DiPKS14 has 50% query cover and is 27% identical and 46% similar to the lovastatin nonaketide synthase enzyme that makes the commercially available drug Lovastatin. Ma *et al.* (2009) have indicated that in invitro biochemical assays utilizing purified full length enzyme, the enoyl reductase and reductase domain of the lovastatin nonaketide synthase were non-functional. Our bioinformatics analysis of the highly similar DiPKS14 polyketide synthase from *Dictyostelium discoideum* indicates that these domains have membrane anchoring hydrophobic stretches. It is

likely that the presence of membrane lipids are required for the proper folding of these domains, in the absence of which they are non-functional. Since the hydrophobic stretches reside within the domains and not in the linker regions we predict that the enzyme to be membrane anchored as opposed to being a transmembrane protein. In the model for the DiPKS14 domain architecture we depict the enzyme to be membrane anchored so as to maintain the continuity of the domain on one side of the membrane. There are no known membrane anchored PKS enzymes identified to date although PKS enzymes are often observed near membranes in vivo (Straight *et al*., 2007; Hoefler *et al*., 2017). We present a schematic model (Figure 10) of the DiPKS14 enzyme as a highly reducing type I polyketide synthase protein with two membrane anchoring regions and a polar-nonpolar bi-domain preceding the catalytic triad in the reductase domain. We predict that DiPKS14 is involved in membrane associated functions.

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