Chapter 2

Biochemical characterization of Dictyostelium phosphopantetheinyl transferases
2.1 **INTRODUCTION**

*Dictyostelium discoideum* (Dicty) is a unicellular amoeba that undergoes multicellular differentiation when faced with starvation. Various small metabolites are known to mediate this morphogenesis (Konijn *et al.*, 1967; Saran, 1999; Sternfeld *et al.*, 1979; Anjard *et al.*, 1998; Serafimidis *et al.*, 2005; Gibson *et al.*, 1988). Two of its important developmental regulating factors (DRFs) – Differentiation Inducing Factor-1 (DIF-1) and 4-methyl-5-pentylbenzene-1,3-diol (MPBD) are of polyketide origin, synthesized by large multifunctional polyketide synthases (PKSs) (Austin *et al.*, 2006; Ghosh *et al.*, 2008). Decoding of the Dicty genome revealed an interesting aspect about this simple organism – presence of an astoundingly large number of type I PKSs (Eichinger *et al.*, 2005). It has been widely speculated that these PKSs could be playing a significant role during its well-programmed unicellular to multicellular transition (Eichinger *et al.*, 2005; Austin *et al.*, 2006; Zucko *et al.*, 2007; Ghosh *et al.*, 2008). However, a key step in understanding the relevance of these large enzymes in Dicty biology is to decipher their mode of activation. PKSs are functionally inactive unless their ACP domains undergo a post-translational modification. This modification is catalyzed by a group of enzymes known as phosphopantetheinyln transferases (PPTases) (Lambalot *et al.*, 1996; Walsh *et al.*, 1997). The modification of carrier domains involves the transfer of a 340 Da phosphopantetheine group derived from coenzyme A (CoA) on to the hydroxyl group of serine residue of ACP domains. PPTases are also required for activating carrier proteins (CPs) of fatty acid synthases (FASs), non-ribosomal peptide synthases (NRPSs), adipate semialdehyde
dehydrogenase (Lamabalot et al., 1996) and 10'-formyl tetrahydrofolate dehydrogenase (Donato et al., 2007).

Conventionally, PPTases have been broadly categorized into two families (Mootz et al., 2001). The prototype for the first family of enzymes is the Sfp protein from Bacillus subtilis. Sfp-like PPTases are approximately 34 kDa enzymes. These proteins exhibit promiscuous capability to post-translationally modify wide range of carrier proteins. The members of second family of enzymes, designated as AcpS, are relatively small (about half the size) and are known to be generally more stringent in choosing carrier proteins. This family also includes a separate subfamily of integrated PPTases involved in fungal FAS activation (Stuible et al., 1997; Fichtlscherer et al., 2000). Several studies in recent years have revealed interesting insights into these enzymes. However, significance of PPTases in developmentally complex organisms is still obscure.

The genome of B. subtilis encodes for AcpS and Sfp, former is involved in fatty acid synthesis and the latter in surfactin production (Mootz et al., 2001). However, in the absence of AcpS, Sfp has been shown to take over its function. This is in contrast to Mycobacterium tuberculosis where the roles of AcpS and Sfp have been reported to be non-redundant in nature (Chalut et al., 2005). The three PPTases from Myxococcus xanthus are categorized into AcpS, that is involved in fatty acid biosynthesis, and two redundant Sfp proteins, which are modifying enzymes of
secondary metabolic pathway (Meiser and Müller, 2008). In certain examples, PPTases are known to be associated specifically with enzyme systems (Li and Piel, 2002; Wang et al., 2001, Huang et al., 2006). In parasites, the function of two PPTases is suggested to be dictated by the architecture of the proteins (Cai et al., 2006). In *Toxoplasma gondii*, the two PPTases have been proposed to cater to the type I and type II FAS systems. Interestingly, in *Cryptosporidium parvum* a single PPTase, Sfp is capable of modifying multifunctional type I PKS and FAS and it does not contain AcpS. *Plasmodium*, which possesses only type II FAS, has AcpS for mediating the activation of CP. In fungi such as *Aspergillus nidulans* one of the three PPTases is an integral component of the FAS α-subunits and another one is suggested to be involved in activation of a mitochondrial FAS (Marquez et al., 2007). The third PPTase is demonstrated to be Sfp-like protein that is responsible for the activation of all its 27 PKSs and 14 NRPSs.

Despite characterization of several different Sfp and AcpS-like PPTases, presently it is not possible to predict their specificity based on computational analysis (Table 2.1). It is therefore important to characterize their biochemical activity and delineate their functional specificities in a case-specific manner.
### Table 2.1 Specificity of the two types of PPTases in different organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>AcpS-like</th>
<th>Sfp-like</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>II</td>
<td>I, II</td>
<td>Sfp can take over the function of AcpS in its absence.</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em></td>
<td>I, II</td>
<td>I</td>
<td>Both PPTases non-redundant.</td>
</tr>
<tr>
<td><em>Streptomyces verticillus</em></td>
<td>I, II</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>II, II</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Myxococcus xanthus</em></td>
<td>II</td>
<td>I</td>
<td>Two Sfp present</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td></td>
<td>I, I</td>
<td></td>
</tr>
<tr>
<td><em>Plasmodium falciparum</em></td>
<td>II</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Toxoplasma gondii</em></td>
<td>II</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td><em>Aspergillus nidulans</em></td>
<td>II</td>
<td>I</td>
<td>One PPTase integrated with cytosolic type I FAS</td>
</tr>
</tbody>
</table>

- **Green**: cytosolic Fatty Acid Synthase
- **Red**: Polyketide Synthase/ Non-Ribosomal Peptide Synthase
- **Blue**: mitochondrial Fatty Acid Synthase
- **I**: Type I ACP
- **II**: Type II ACP

In this study, we have investigated the functional role of two PPTase homologues in *Dictyostelium* biology. Here, we demonstrate that the two PPTases, DiAcpS and DiSfp are functionally discrete and non-redundant in nature. Furthermore,
biochemical studies unambiguously show that DiSfp is required for the activation of multifunctional PKS/FAS, whereas, DiAcpS can modify only the stand-alone ACP. We also show that both PPTases are expressed during all stages of Dicty life cycle. Our studies thus establish the important role of these proteins in *Dictyostelium*.

### 2.2 MATERIALS AND METHODS

#### 2.2.1 Materials

Synthetic oligonucleotides were obtained from Sigma GENOSYS. DNA polymerase, restriction and modifying enzymes were procured from Stratagene and New England Biolabs (NEB). The cloning and expression vector systems were obtained from Stratagene and Novagen. DNA purification kits and Ni$^{2+}$-NTA agarose resin were procured from Qiagen. Radiolabeled fatty acyl CoA substrates were obtained from Perkin Elmer Life Sciences and American Radiolabeled Chemicals. All other chemicals used were of analytical grade.

#### 2.2.2 Methods

**2.2.2.1 Genomic DNA isolation from Dictyostelium**

*Dictyostelium* AX2 cells were used for the entire study and grown at 22°C in HL-5 axenic medium. For genomic DNA isolation, $10^7$ to $10^9$ cells were harvested and washed twice in 1X KK2 buffer. Cell pellet was then washed with DB buffer and mixed gently with 5 volumes of RLB buffer for nuclei extraction. Following this, cells were pelleted again and washed with cold RLB buffer. This was followed by re-
suspending the pellet in 500 µl of RLB buffer and incubation at 37°C for 5 minutes. After this Buffer 1 was added along with RNase and suspension was mixed gently. Subsequently, Buffer 2 was added with proteinase K and the mix was incubated at 65°C and 37°C each for 1 hr. Phenol: chloroform extraction was eventually done and DNA was precipitated with 100% ethanol. Following ethanol wash, pellet was air dried and re-suspended in desired volume of Tris 10 mM pH 8.0.

2.2.2.2 Polymerase chain reaction (PCR)

The PCR reactions contained 2.5 mM dNTPs, 50-500 pmol of the forward and the reverse primer, 1X concentration of buffer along with *Amplitaq* or *Phusion* enzyme in a 50 µL reaction volume. The enzyme was added after an initial hot start at 98°C for 2 min. The PCR amplification was carried out in 30 cycles. Lower annealing temperatures of 48-57°C were used for amplification. The last extension cycle was for 10 min at 68°C or 72°C. The details of the primers used for various PCR reactions are listed in Table 2.2. To facilitate cloning in expression vectors, restriction endonuclease sites were suitably engineered in the oligonucleotide primers designed for PCR reaction.
Table 2.2: Sequences of primers used in this study. The restriction enzyme recognition sites are highlighted in bold.

### 2.2.2.3 Agarose gel electrophoresis

DNA fragments were resolved on 1% agarose gel containing 0.5 μg/mL of ethidium bromide at 10 V/cm in 1x TAE buffer. The samples containing 1x gel loading dye (containing bromophenol blue as tracking dye) were subjected to electrophoresis and the DNA bands were visualized on UV transilluminator at 312 nm. The size of DNA fragments were estimated by comparing their mobility with that of the DNA fragments in 1kb DNA ladder (NEB) and 100bp DNA ladder (NEB).
2.2.2.4 Purification of PCR products

After electrophoretic separation of DNA, the desired band was excised from the agarose gel with a sterile scalpel. QIAquick DNA binding columns were used to purify DNA using the protocol provided with QIAquick gel extraction kit (Qiagen, Germany).

2.2.2.5 Phosphorylation of PCR amplified DNA

In order to facilitate blunt-end ligations, the PCR product was phosphorylated using T4 polynucleotide kinase in the presence of T4 DNA ligase buffer (NEB) in a 50 μL reaction volume. The reactions were incubated at 37°C for 45 min and then purified using the Qiagen DNA purification kit.

2.2.2.6 Restriction endonuclease fragmentation analysis

The restriction endonuclease fragmentation pattern of plasmid DNA was analyzed by using specific enzymes. One unit of enzyme was used for fragmentation of 1 μg of DNA in the appropriate reaction buffer provided by the manufacturer. Analytical reactions were carried out in a final volume of 20 μL at 37°C for 1-2 hrs. Preparative reactions were carried out under similar conditions for 5-10 hrs.
2.2.2.7 Dephosphorylation of vector DNA

After restriction endonuclease treatment, the linearised vector was treated with calf intestinal alkaline phosphatase (CIAP) in order to reduce the probability of self-ligation. 1 μL of CIAP was added after the restriction endonuclease reaction was complete. The reactions were incubated at 37°C for 1 hr and then purified using the Qiagen DNA purification columns according to manufacturer’s protocol.

2.2.2.8 Cloning of PCR fragments into pBluescript SK (+)

T4 DNA ligase was used to ligate the insert with the linearised vector DNA in a 10 μL reaction containing 1X T4 DNA ligase buffer. The reaction was incubated at 16°C for 4 hrs and transformed into XL-1-Blue competent cells.

2.2.2.9 Preparation of competent cells

The chemical-competent cells were prepared for various strains used in this study. 100 mL of LB culture medium was inoculated with 1% inoculum from an overnight grown culture and incubated in a 37°C incubator-shaker till the $A_{600}$ reached 0.5 units. Cell growth was stopped by incubating the flask on ice for 10 min. The culture was harvested by centrifugation at 3000 rpm for 15 min at 4°C. The cells were resuspended in 20 mL of chilled TF1 buffer and incubated on ice for 20 min. The cells were pelleted again by centrifugation at 3000 rpm for 15 min at 4°C. The pellet was resuspended in 4 mL of TF2 buffer. These competent cells were aliquoted and
stored at -80°C till further use. Competent cells were prepared for the XL-1 Blue, BL21-DE3, BAP1, BL21-CodonPlus (DE3)-RP strains of *Escherichia coli* (*E. coli*).

### 2.2.2.10 Transformation and screening of prospective clones

10 μL of the ligation mixture or 0.1 μg of plasmid DNA was used to transform 50 μL of the competent cells. The cells were incubated on ice for 30 min. after addition of the DNA. A heat shock was given at 45°C exactly for 90 seconds. After incubation on ice for 2-3 min, 500 μL of LB medium was added and the cells were incubated at 37°C with shaking for 1 hr. The contents were then spread on LB plates containing respective antibiotics i.e. ampicillin or carbenicillin for plasmids with pET-21c(+) and pBluescript SK(+) backbone and kanamycin for plasmids with pET-28a(+). The plates were allowed to air dry and incubated at 37°C for 12-16 hrs.

Plasmid pBluescript SK(+) (pBS) linearised by treatment with restriction endonucluease *EcoRV* was used as vector for cloning PCR products. The *EcoRV* restriction site is a part of the multiple cloning site (MCS) that lies within the coding region of the β-galactosidase (*lacZ*) gene of this vector. Successful ligation of a foreign DNA into the *EcoRV* site abolishes production of functional β-galactosidase which can degrade X-gal to give blue coloured colonies. The preliminary screening of recombinant colonies was performed by blue-white selection on LB agar medium containing IPTG and X-gal. The white colonies obtained were grown to isolate the plasmid, which was then further analyzed by restriction fragmentation to confirm the
recombinant clones. In certain cases, either TOPO (for directional cloning) or TA cloning vectors were used for cloning the PCR products.

2.2.2.11 Plasmid isolation

The plasmid DNA was purified using the alkaline lysis method (Sambrook et al., 1989). The overnight grown culture was harvested by centrifugation and then resuspended in P1 buffer [50 mM Tris-HCl (pH: 8.0), 10 mM EDTA, 100 µg/µL RNase A]. The cells were then lysed by addition of P2 buffer containing 200 mM sodium hydroxide and 1% SDS. The tubes were incubated at room temperature for 3-5 min. The plasmid DNA was then renatured by adding the neutralizing P3 buffer [3 M potassium acetate (pH: 5.5)] while the genomic DNA and the proteins precipitated. The plasmid DNA obtained in supernatant after centrifugation at 13,000 rpm for 10 min was precipitated by addition of 0.7 volume of isopropanol. The plasmid was pelleted by centrifugation and then washed with ice cold 70% ethanol to remove the salts. The plasmid was then air-dried and resuspended in 10 mM Tris-HCl (pH: 8.0) or autoclaved milli Q (MQ) water for further use.

2.2.2.12 Identification of domain boundaries

Primary sequences of various genes were obtained from Dictybase (http://www.dictybase.org) and National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). Domain boundaries for various proteins were
identified by sequence based analysis using NRPS-PKS search tool, a program
developed for identification and analysis of NRPS and PKS domains
(http://www.nii.res.in/nrps-pks.html) (Yadav et al., 2003). Further analysis was
performed by using NCBI-Conserved Domain Search (NCBI-CDS) program

2.2.2.13  Expression and purification of PPTases and ACPs

The plasmids carrying genomic sequences corresponding to the PPTases or ACPs
were transformed in expression strain of E. coli for over-expression of proteins.
Protein expression was first checked on small scale at different temperature conditions
and IPTG concentrations in 5 mL cultures. The E. coli cells harbouring the
recombinant plasmid clones were grown in LB broth with 50 μg mL\(^{-1}\) of the
appropriate antibiotic (carbenicillin/kanamycin) at 37°C or 30°C until an O.D\(_{600}\) of
0.6 was reached. The culture was induced with 0.1-0.5 mM of IPTG and further
incubated in a shaker at 22°C or 18°C for 8-12 hrs. The culture conditions that
resulted in maximal expression were chosen to set up 2 L batch cultures for large
scale protein purification. After harvesting, the cells were resuspended in lysis buffer
(50 mM Tris pH: 8.0, 10% glycerol) and disrupted using french press at 1100 psi
pressure. Cell debris was removed by centrifugation at 50,000 g for 40 min at 4°C.
0.75 ml L\(^{-1}\) of Ni\(^{2+}\)-NTA slurry was added to the supernatant and incubated at 4°C for
1 hr. The mixture was loaded onto a column working under gravity flow. The resin
was washed with wash buffer (50 mM phosphate buffer pH: 8.0, 10% glycerol and 5
mM imidazole) till all unbound proteins were removed. The protein was eluted using elution buffers containing increasing concentration of imidazole. Fractions containing the proteins of interest were pooled and 1 mM TCEP was added. The protein of interest was collected and stored at -80°C for further use after adding 1 mM TCEP.

2.2.2.14 Estimation of protein concentration

Protein estimation was carried out by modification of the Folin-Lowry’s method (Lowry et al., 1951). Various aliquots of protein were taken and diluted to a final volume of 100 μL. To this, 100 μL of 1 N NaOH was added and incubated at 100°C for 10 min to denature the proteins. The denatured proteins were cooled to room temperature and 1.0 mL of alkaline copper sulphate reagent was added. After incubation at room temperature for 10 min, 0.1 mL of Folin-Ciocalteu reagent was added and further incubation was carried out for 30 min at 37°C. The absorbance was monitored at 620 nm. The unknown concentrations were estimated on the basis of a standard curve which was plotted using various concentrations of bovine serum albumin (BSA).

2.2.2.15 Protein sequencing by mass spectrometry

The protein was subjected to SDS-PAGE analysis and was visualized by staining with Coomassie Brilliant Blue R 250 followed by destaining of the gel. All the steps were carried out wearing powder free gloves. The protein band was excised from the gel.
and cut into small pieces with an unused scalpel. The gel pieces were then transferred into a 0.5 mL microcentrifuge tube and 200 μL of destain [25 mM ammonium bicarbonate/acetonitrile/water (1:1:2, v/v) solution] was added to the tube. The tube was incubated in a thermomixer at 37°C, 800 rpm for 20 min after which the destain was removed. After the last destaining step 50 μL of water/acetonitrile (1:1, v/v) was added to each tube and incubated in a thermomixer at 30°C, 800 rpm for 15 min. Acetonitrile was removed by aspiration and discarded and the remaining water was removed from the gel pieces by vacuum concentration till the gels pieces dehydrated to a white opaque mass. Now 50 μL of 10 mM DTT was added to each sample and the samples were then incubated on thermomixer at 45°C, 800 rpm for 45 min. 50 μL of 50 mM iodoacetamide dissolved in 25 mM ammonium bicarbonate was added and the samples were incubated in dark at ambient temperature for 30 min. The supernatant was discarded, 50 μL of water/acetonitrile (1:1, v/v) was added to each tube and samples were incubated on the thermomixer at 30°C, 800 rpm for 15 min. The residual fluid was removed and the samples were incubated with 50 μL of acetonitrile at ambient temperature for 5 min. 50 μL of 25 mM ammonium bicarbonate was added and the samples were incubated at ambient temperature for 15 min. The supernatant was discarded and the samples were dried by vacuum concentration for 30 min. Lyophilized trypsin (Promega) was resuspended in 100 μl of trypsin buffer to obtain 0.2 μg/μL stock. The required amount was diluted in 10 mM ammonium bicarbonate to obtain 10 ng/mL working concentration. This diluted trypsin solution was added to the tube such that the gel pieces were completely
submerged. The tubes were first incubated on ice for 30 min. Subsequently 10 μL of 10 mM ammonium bicarbonate was added and samples were incubated at 37°C for 16 hrs. The peptides were extracted in 20 μl extraction buffer (water/acetonitrile/trifluoroacetic acid, 0.5:0.49:01, v/v/v). The supernatant was collected and stored at -20°C till further MALDI-MS analysis.

The matrix α-cyano hydroxyl cinnamic acid was dissolved in acetonitrile/water/trifluoroacetic acid (0.5:0.5:0.01, v/v/v) to a final concentration of 5 mg/mL. The trypsinised samples were mixed with matrix in a ratio of 1:1, a total volume of 1 μL was spotted on a clean MALDI plate. The calibration standard 4700 mix (Applied Biosystems) was mixed with matrix in a ratio of 1:24, v/v and 1 μL of this was spotted on the calibration spots of the same MALDI plate. MS and tandem MS (MS/MS) analysis data was submitted to MASCOT-search in order to identify the proteins by peptide mass fingerprinting.

2.2.2.16 PPTase gel-binding assay
Radioactive assays were set up with 57 μM of apo-ACP, 20 μM PPTase, 12.5 mM MgCl₂, 45 μM [1-14C] Acetyl CoA and 50 mM Tris-Cl (pH 8.0), in a final volume of 20 μL. Reaction was quenched directly by adding non-reducing SDS dye after incubation of 20 minutes at 30°C. Samples were loaded on SDS-PAGE, gel was dried and analyzed using a phosphorimager (BAS5001).
2.2.2.17 HPLC-Mass spectrometry coupled assays for PPTase

**enzymatic activity**

Phosphopantetheinylation assay was set up with 54 μM apo-ACP, 34 μM DiAcpS/DiSfp, 12.5 mM MgCl₂, 250 μM CoA and 50 mM Tris-Cl (pH 8.0), in a final volume of 75 μL. Reaction was quenched with 50 mM EDTA after 60 minutes of incubation at 37°C. 50 μL of this reaction mix was then loaded onto Phenomenex, C18 reverse-phase HPLC column and eluted with 20 minutes linear gradient from 12% ACN to 90% ACN in H₂O with 0.1% CF₃CO₂H. The eluted products were concentrated, resuspended in 50% ACN and 0.1% CF₃CO₂H and then analyzed on 4800 MALDI TOF/TOF Analyzer.

2.2.2.18 Kinetic analysis

Kinetic parameters for DiAcpS and DiSfp were determined with 20 μM of either of the PPTase, and 5 – 45 μM [1-¹⁴C] acetyl CoA. Concentration of mycobacterial PKS2 and Rv1344 was fixed at 54 μM. Radiolabeled ACP was quantified using phosphorimager (BAS5001). All the experiments were carried out in triplicates and standard deviations were estimated by using GraphPad.
2.2.2.19 Construction of PPTase and DiPKS37 gene knockout cassettes

Knockouts were constructed as described by Kuwayama et al. Blasticidin resistance (Bsr) cassette was cloned between the XbaI-HindIII sites in pBS from pUC-Bsr vector. Upstream region of the respective PPTases were then cloned as 5' flanks between NotI-XbaI sites. Similarly, downstream regions were cloned as 3' flanks between HindIII-KpnI sites. For constructing dipks37 knockout cassette, regions around its ACP domain were selected. This construct was then digested with NotI and KpnI to release the entire knockout cassette (Figure S1). The digested product was column-purified and precipitated. Linear cassette was then transformed into Dictyostelium amoeboid cells.

2.2.2.20 Transformation of Dictyostelium cells with knockout cassettes for generation of null mutants

Dictyostelium discoideum AX2 cells were grown in a petridish in HL5 medium till they attained confluency. Approximately $10^7$ cells were harvested by centrifugation and washed with ice cold 1X KK2 buffer followed by H50 buffer. Subsequently cell pellet was resuspended in 100 µL H50 buffer and the suspension was gently mixed with the disruption cassette at 4°C in a 0.1 mm electroporation cuvette. Electroporation was performed for 1 ms at 650 V and 25 µF with Gene Pulser Xcell (Biorad), two pulses were applied at 30 s interval. After electroporation 100 µL of H50 was added in the cuvette, and the content was transferred into a petridish.
containing 2 μL of "healing solution". Following this, petridish was shaken at 40 rpm for 15 min. at 22°C. After 15 min., 37 mL medium was added to the petridish and contents mixed thoroughly and gently. 100 μL of this suspension was then aliquoted into four 96-wells plates and cells were allowed to recover for 24 hrs. 24 hrs. after electroporation, 5 μg/mL of blasticidin was added and the cells were constantly monitored. Surviving cells were gradually exposed to increasing antibiotic concentrations, upto 100 μg/mL. Clonal population resistant to highest antibiotic concentration were PCR screened for homologous recombination and confirmation of null mutant clones. Primers used for checking recombinants are listed in Table 2.3
<table>
<thead>
<tr>
<th>Primer description</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>*disfp 5' genomic region</td>
<td>RSG 2001 5' TTCAATTATTTAATTATTCCTGGCAAAA 3'</td>
</tr>
<tr>
<td>*disfp 5' flank forward primer</td>
<td>RSG 1258 5' CACCCAGGCTCACACACACCAATATCTCATAT 3'</td>
</tr>
<tr>
<td>*disfp 5' flank reverse primer</td>
<td>RSG 1259 5' GTCTAGAAAAAACCCCCATGAAATGAAATATAA 3'</td>
</tr>
<tr>
<td>*diacps 5' genomic region</td>
<td>RSG 1999 5' TGATAATTATATATCAATAATATTCTGGTTAAC 3'</td>
</tr>
<tr>
<td>*diacps 5' flank forward primer</td>
<td>RSG 1363 5' CGGCGGCCGCTGATTATAAAATTACTAAAATCTTCT 3'</td>
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<td>*diacps 5' flank reverse primer</td>
<td>RSG 1364 5' GTCTAGATCTCTGATATCTTTATCAATATCATTCC 3'</td>
</tr>
<tr>
<td>*dips37 5' genomic region</td>
<td>RSG 1952 5' CGCCGAATAATATCCAAATTATGGAATG 3'</td>
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<tr>
<td>*dips37 3' genomic region</td>
<td>RSG 1953 5' CGGAGTACCACTCGAAGAAGGG 3'</td>
</tr>
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<td>*dips37 5' flank forward primer</td>
<td>RSG 1911 5' CGGCGGGGGCGCCTTTAAATCTGATTGTCGTATATG 3'</td>
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<tr>
<td>*dips37 5' flank reverse primer</td>
<td>RSG 1912 5' CGCTCTAGGATGACATGATCTAAGGCTG 3'</td>
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<tr>
<td>Blasticidin cassette forward primer</td>
<td>RSG 1551 5' GAATGGCAAGTTAGTCAAATACGTCTCTTCC 3'</td>
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<tr>
<td>Blasticidin cassette reverse primer</td>
<td>RSG 1550 5' CGCTACTCTACTTTAATGATCTCGTTG 3'</td>
</tr>
</tbody>
</table>

Table 2.3 Primers used for checking homologous recombination in *Dictyostelium* mutant strains.

2.2.2.21 *Dictyostelium small scale genomic DNA isolation*

Dicty cells were collected in eppendorf tubes and pelleted down at 1000 rpm for 10 s. Subsequently cells were washed twice with cold autoclaved milli Q water and resuspended in 100 µL PCR buffer [10 µL of 10X Taq reaction buffer, 1 µL of 5 mg/mL proteinase K and 2.5 µL of 20% NP-40]. The reaction was then incubated at 56°C for 45 min. and 100°C for 10 min. This was then stored in -20°C until further use.
2.2.2.22  **Starvation-induced development of Dictyostelium**

Dicty cells were harvested by centrifugation at 3000 rpm for 2 min. and washed with 1X KK2 buffer thrice. The pellet was resuspended in 1X KK2 buffer and the cell suspension was poured on to non-nutrient agar (NNA) plate. After cold synchronization for 9 hrs., the plates were incubated at 22°C for development.

2.2.2.23  **RNA isolation from Dictyostelium and cDNA synthesis**

RNA for RT-PCR was extracted from amoeboid cells and developmental stages using TRI reagent and was subsequently treated with DNAse and column-purified using QIAgen kit. RT-PCR was performed using Invitrogen kit with oligodT primer and gene expression checked using gene-specific PCR primers. RT-PCR conditions used were as follows: One cycle of 60 min at 50°C for reverse transcription and one cycle of 30 sec at 98°C, followed by 30 cycles of 15 sec at 98°C, 30 sec at 45°C annealing, and 30 sec at 68°C for extension. The details of the primer sets used are included in Table 2.4. IG7 (mitochondrial large rRNA) was used as the RT-PCR control.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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<tr>
<td>disp</td>
<td>RSG 1135 5'ACCCATGAGTAGATTGATATCTTGTACATACGCA 3'</td>
<td>RSG 1076 5'ACGAGGCTTACCTTATATTCTTAATCTG 3'</td>
</tr>
<tr>
<td>diacps</td>
<td>RSG 1354 5'ACATGGTAGATACATATCTAATTTTAAAAGGC 3'</td>
<td>RSG 1734 5'TTAAATTCTACTGCATTGATATCTAA 3'</td>
</tr>
<tr>
<td>IG7</td>
<td>RSG 1440 5'GGATTCTGAAAAATGCCCAAC 3'</td>
<td>RSG 1440 5'GTCTCTGTGTAAAGAAAGG 3'</td>
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**Table 2.4 List of primers used for RT-PCR studies.**
2.3 RESULTS

2.3.1 Bioinformatics analysis for identification of Dictyostelium PPTases

2.3.1.1 Identification of disfp and diacps

We performed BLAST searches with *Streptococcus pneumoniae* AcpS and *B. subtilis* Sfp on Dictybase (www.dicybase.org) to identify PPTase-like sequence(s) in the Dicty genome. We decided on these templates because structures of *S. pneumoniae* AcpS and *Bacillus* Sfp have already been elucidated and the latter has been extensively characterized and is considered as the prototype of Sfp-like PPTases. Our analysis revealed the presence of two protein sequences with considerable homology. DDB0304576 (DiSfp - previously annotated as DDB0186752) exhibits 49% similarity with *B. subtilis* Sfp, whereas, DDB0349466 (DiAcpS - previously annotated as DDB0217726) shows 45% similarity with *S. pneumoniae* AcpS (Figs. 2.1 and 2.2).
Figure 2.1 Sequence BLAST on Dictybase using *B. subtilis* Sfp identifies only one protein (DDB0304576) with considerable homology.

Figure 2.2 Sequence BLAST on Dictybase using *S. pneumoniae* AcpS identifies only one protein (DDB0349466) with good homology.
Apart from these sequences, no other homologous sequences could be identified from the genome. Sfp and AcpS like PPTases from apicomplexans and other organisms contain two conserved motifs as determined by PSSM analysis of 312 sequences. Both the putative Dicty PPTases show these characteristic motifs - [IV]G[ITV]D[ILV][VE] and W[CA][AL]KEAxK. In addition, DiSfp also contains the FNxSH motif that is typically present in Sfp type PPTases (Cai et al., 2006) (Fig. 2.3). Comparative analysis of these protein sequences based on the crystal structures of *B. subtilis* AcpS and Sfp (Reuter et al., 1999; Parris et al., 2000) also showed conservation of key residues. This includes R16 and R23 residues in DiAcpS involved in recognition of ACP and so also the amino acids D8, F25, R28, E58 and F74 (numbering corresponds to *Bacillus* AcpS) reported to be involved in binding of CoA and Mg\(^{2+}\). Careful analysis of DiSfp sequence also revealed the presence of residues – H118, S117, K52 and K193 which have been shown in *B. subtilis* Sfp to be involved in CoA binding.
Figure 2.3 Sequence alignment of DiSfp with *B. subtilis* Sfp (a), and DiAcpS with *B. subtilis* AcpS (b) shows the presence of motifs characteristic of PPTases and conservation of key residues.
2.3.1.2 Phylogenetic analysis of Dictyostelium PPTases

Dendrogram-based phylogenetic analyses of Dicty PPTase sequences with other lower eukaryotes, including fungi and protozoan parasites readily provided their classification in two distinct groups of AcpS-like and Sfp-like sequences (Fig. 2.4). In order to understand the relevance of the two putative PPTases from Dicty, we decided to carry out genetic and biochemical investigations.

Figure 2.4 Phylogenetic analysis segregates the Dicty PPTases into two different groups.
2.3.2 Functional significance of Dicty PPTases in its biology

Three different approaches were undertaken to study the role of PPTases. Firstly, temporal changes in the expression of genes through different developmental stages were analyzed. Secondly, we attempted to disrupt these genes by using homologous recombination and finally we performed direct biochemical assays to delineate exact functions of these proteins.

2.3.2.1 RT-PCR analysis of disfp and diacps

We examined the expression profiles of disfp and diacps to determine if the genes were temporally regulated in Dicty. Primers for performing RT-PCR were designed from the exon 2 region in either case. Eight synchronous stages were chosen for analysis: amoeboid, T0 (0 hrs. after starvation), streaming, loose aggregate, mound, slug, early culminating and fruiting body. RNA was isolated from each of these stages and the yield varied between 0.3 μg/μL and 1 μg/μL. cDNA synthesized from 1 μg RNA samples were then used for setting up RT-PCR studies.

Notably, expression of both the genes could be detected during the vegetative as well as various developmental stages of the organism (Fig. 2.5). In order to elucidate functional overlap between the two PPTases, we decided to create genetic-knockouts of the individual genes.
Figure 2.5 Expression profiles of disfp and diacps through different developmental stages of Dicty. Stages analyzed were amoeboid (1), 0 h after starvation (2), streaming (3), loose aggregate (4), mound (5), slug (6), early culminant (7) and fruiting body (8). L1 = 1kb ladder, L2 = 100bp ladder. Expected amplicon size for: disfp – 447 bp, diacps – 444 bp and IG7 (normalization control) – 852 bp.
Cloning of PPTase and dipks37 knockout constructs

Various constructs for disfp and diacps were generated. A common strategy followed for generating these plasmids is shown in Fig. 2.6. Two independent constructs for both genes involved varying 5' and 3' flanking regions. Dipks37 knockout construct was also generated using earlier reported sites (Austin et al., 2006) as a control for the disruption technique used for our studies.

![Diagram showing cloning strategy](image)

Figure 2.6 Cloning strategy used in this study for creating gene knockout constructs. The orange coloured bar represents the gene and the dotted lines represent the sequences on either side of the gene.
In the case of first *disfp* knockout construct, the 5' and 3' flank regions of the gene were amplified from the genomic DNA. The 1074 bp 5' flank PCR product was first cloned into TOPO vector (pCR419), followed by cloning into the PBS-Bsr vector (PBS vector with Bsr cassette integrated in it) to give pDN3. On the other hand, the 1054 bp 3' flank product was cloned in the TA vector (pCR420). This fragment was subsequently cloned into the pDN3 plasmid to yield the *disfp* knockout construct (pDN4). For the second knockout construct, different primer sets were used for the amplification of both the flank regions. The 967 bp 5' flank PCR product was directly cloned into PBS-Bsr vector and the resultant plasmid was named pDN8. The corresponding 3' flank region was cloned into this plasmid to give the full gene knockout construct (pDN9). All clones were confirmed using restriction enzyme fragmentation analysis (Fig. 2.7). The fragments obtained matched with the expected fragmentation pattern tabulated in Table 2.5.
Figure 2.7 Restriction fragmentation analysis of disfp knockout construct. Expected fragment sizes in bp are indicated in parentheses.

1. 100bp ladder
2. 1kb ladder
3. pCR419 NotI, XbaI (2580, 1074)
4. 100bp ladder
5. 1kb ladder
6. pDN3 NotI, XbaI (4358, 1074)
7. 1kb ladder
8. pDN3 NotI, HindIII (2958, 2474)
9. pCR420 KpnI, HindIII (3015, 1054)
10. 100bp ladder
11. 1kb ladder
12. 1kb ladder
13. pDN4 NotI, XbaI (5412, 1074)
14. 1kb ladder
15. pDN4 KpnI, HindIII (5432, 1054)
16. 1kb ladder
17. pDN4 XbaI, HindIII (5086, 1400)
18. pDN4 XbaI, KpnI (4032, 2454)
19. 1kb ladder
20. 1kb ladder
21. pDN8 NotI, XbaI (4358, 1074)
22. 1kb ladder
23. pDN9 HindIII, KpnI (5325, 1040)
24. pDN9 NotI, KpnI (2958, 3407)
25. 1kb ladder
<table>
<thead>
<tr>
<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' flank (#1) in TOPO</td>
<td>pCR419</td>
<td>NotI, XbaI 2580, 1074</td>
</tr>
<tr>
<td>3' flank (#1) in TA</td>
<td>pCR420</td>
<td>KpnI, HindIII 3015, 1054</td>
</tr>
<tr>
<td>5' flank (#1) in pBS-Bsr</td>
<td>pDN3</td>
<td>NotI, XbaI 4358, 1074</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NotI, HindIII 2958, 2474</td>
</tr>
<tr>
<td>disfp full knockout construct (#1)</td>
<td>pDN4</td>
<td>NotI, XbaI 5412, 1074</td>
</tr>
<tr>
<td></td>
<td></td>
<td>KpnI, HindIII 5432, 1054</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XbaI, HindIII 5086, 1002</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XbaI, KpnI 4032, 2454</td>
</tr>
<tr>
<td>5' flank (#2) in pBS-Bsr</td>
<td>pDN8</td>
<td>NotI, XbaI 4358, 1074</td>
</tr>
<tr>
<td>disfp full knockout construct (#2)</td>
<td>pDN9</td>
<td>KpnI, HindIII 5325, 1040</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NotI, KpnI 2958, 3407</td>
</tr>
</tbody>
</table>

Table 2.5 Restriction digestion pattern of disfp knockout construct. Fragment sizes are in bp.
Similarly, 5′ and 3′ flank regions (1054 bp and 1284 bp respectively) for generation of *diacps* knock-out construct were PCR amplified and cloned into TA vector separately and the clones were named pCR421 and pCR422 respectively. Eventually 5′ flank was cloned into pBS-Bsr vector (pDN6), followed by insertion of the 3′ flank into this vector. The *diacps* knock-out construct thus generated would be referred to as pDN7. The second construct (pDN11) was created with different set of primers. Cloning involved insertion of the 991 bp 5′ flank (to yield the plasmid – pDN10), followed by 3′ flank (910 bp) into the pBS-Bsr vector directly. Confirmatory tests for checking clones were performed using restriction enzymes (Fig. 2.9; Table 2.6).
Figure 2.9  Restriction fragmentation analysis of *diacps* knockout construct. Expected fragment sizes in bp are indicated in parentheses.

1. 100bp ladder
2. 1kb ladder
3. pCR421 *NotI*, *XbaI* (3015, 1054)
4. 1kb ladder
5. pCR422 *Kpnl*, *HindIII* (3015, 1284)
6. pDN6 *NotI*, *XbaI* (4358, 1054)
7. 1kb ladder
8. 1kb ladder
9. pDN7 *Kpnl*, *HindIII* (5412, 1149)
10. 1kb ladder
11. pDN7 *XbaI*, *HindIII* (5296, 1400)
12. pDN7 *XbaI*, *Kpnl* (4012, 2684)
13. 1kb ladder
14. 1kb ladder
15. pDN10 *NotI*, *XbaI* (4358, 991)
16. 1kb ladder
17. pDN11 *HindIII*, *Kpnl* (5349, 803)
18. pDN11 *NotI*, *Kpnl* (2958, 3194)
19. 1kb ladder
<table>
<thead>
<tr>
<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' flank (#1) in TA</td>
<td>pCR421</td>
<td>NotI, XbaI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3015, 1054</td>
</tr>
<tr>
<td>3' flank (#1) in TA</td>
<td>pCR422</td>
<td>KpnI, HindIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3015, 1284</td>
</tr>
<tr>
<td>5' flank (#1) in pBS-Bsr</td>
<td>pDN6</td>
<td>NotI, XbaI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4358, 1054</td>
</tr>
<tr>
<td>diacps full knockout construct (#1)</td>
<td>pDN7</td>
<td>KpnI, HindIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5412, 1149</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XbaI, HindIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5296, 1400</td>
</tr>
<tr>
<td></td>
<td></td>
<td>XbaI, KpnI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4012, 2684</td>
</tr>
<tr>
<td>5' flank (#2) in pBS-Bsr</td>
<td>pDN10</td>
<td>NotI, XbaI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4358, 991</td>
</tr>
<tr>
<td>diacps full knockout construct (#2)</td>
<td>pDN11</td>
<td>KpnI, HindIII</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5349, 803</td>
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<tr>
<td></td>
<td></td>
<td>NotI, KpnI</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2958, 3194</td>
</tr>
</tbody>
</table>

Table 2.6 Restriction digestion pattern of diacps knockout construct. Fragment sizes are in bp.
Dipks37 knockout construct (pDN13) was generated by inserting the 5' amplified region (867 bp) into pBS-Bsr vector (pDN12) and then, the 1196 bp 3' region. Analysis of clones at various steps is represented in Fig. 2.10 and Table 2.7.

![Image of gel electrophoresis](image)

**Figure 2.10** Restriction fragmentation analysis of *dipks37* knockout construct. Expected fragment sizes in bp are indicated in parentheses.

1. 1kb ladder
2. 100 bp ladder
3. pDN12 *NotI, XbaI* (4358, 867)
4. 1kb ladder
5. pDN13 *NotI, XbaI* (5554, 867)
6. 1kb ladder
7. pDN13 *HindIII, KpnI* (5225, 1196)
8. pDN13 *NotI, KpnI* (3432, 2958)
9. 1kb ladder

<table>
<thead>
<tr>
<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5' flank in pBS-Bsr</td>
<td>pDN12</td>
<td><em>NotI, XbaI</em> 4358,867</td>
</tr>
</tbody>
</table>

**Table 2.7** Restriction digestion pattern of *dipks37* knockout construct. Fragment sizes are in bp.
2.3.2.3 Transformation of knockout constructs and screening for mutants

We adopted standard homologous recombination techniques by using blasticidin as the selectable marker. After transformation of the linear cassette, the Dicty amoeboid cells were aliquoted into 96 x 4 wells. On systematic increase of antibiotic selection, ~20% wells showed growth of Dicty cells in the case of disfp (pDN4). For diacps knockout (pDN7) transformants, survival rate was ~ 50%. PCR-based analysis was performed on 96 disfp wells and 124 diacps wells to confirm homologous recombination. However, in all the cases clones were found to be non-homologous recombinants (Fig. 2.11 and Table 2.8). This process was repeated and dipks37 gene disruption cassette (from pDN13) was taken as the control, since a genetic knockout of this has already been described in literature (Austin et al., 2006). Another round of recombination experiments provided only non-homologous recombinants for disfp and diacps. A similar strategy provided five positive clones for Δdipks37 knockout (Fig. 2.11 and Table 2.8).
Figure 2.11 Strategy for confirming homologous recombination of respective knockout constructs in Dicty. Each lane in gel pictures is marked by the combination of primers used (described in Table 2.8).

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer combination used</th>
<th>Primer number</th>
<th>Expected size (bp)</th>
<th>Observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>*disfp</td>
<td>1 + 2</td>
<td>RSG 2001 + RSG 1550</td>
<td>1701</td>
<td>x</td>
</tr>
<tr>
<td>*disfp</td>
<td>1 + 3</td>
<td>RSG 2001 + RSG 1259</td>
<td>1308</td>
<td>v</td>
</tr>
<tr>
<td>*disfp</td>
<td>2 + 4</td>
<td>RSG 1550 + RSG 1258</td>
<td>1360</td>
<td>v</td>
</tr>
<tr>
<td>&gt;dico ps</td>
<td>1 + 2</td>
<td>RSG 1999 + RSG 1550</td>
<td>1764</td>
<td>x</td>
</tr>
<tr>
<td>&gt;dico ps</td>
<td>1 + 3</td>
<td>RSG 1999 + RSG 1364</td>
<td>1371</td>
<td>v</td>
</tr>
<tr>
<td>&gt;dico ps</td>
<td>2 + 4</td>
<td>RSG 1550 + RSG 1363</td>
<td>1384</td>
<td>v</td>
</tr>
<tr>
<td>*dipks37</td>
<td>1 + 2</td>
<td>RSG 1952 + RSG 1550</td>
<td>1661</td>
<td>v</td>
</tr>
<tr>
<td>*dipks37</td>
<td>1 + 3</td>
<td>RSG 1952 + RSG 1912</td>
<td>1259</td>
<td>v</td>
</tr>
<tr>
<td>*dipks37</td>
<td>2 + 4</td>
<td>RSG 1550 + RSG 1911</td>
<td>1260</td>
<td>v</td>
</tr>
<tr>
<td>*dipks37</td>
<td>5 + 6</td>
<td>RSG 1953 + RSG 1551</td>
<td>2352</td>
<td>v</td>
</tr>
</tbody>
</table>

Table 2.8 Primer combinations used for verifying homologous recombinants.
In order to re-confirm our observation, a third round of \textit{disfp} and \textit{diacps} genetic knockouts was attempted with different constructs (pDN9 and pDN11 respectively) along with \textit{dipks37}. Yet again only non-homologous recombinants could be obtained for \textit{disfp} and \textit{diacps}. On the other hand, \textit{dipks37} knockout yielded 25\% homologous recombinants. These studies furnished an indirect support that both the PPTases could be important during the initial vegetative growth. Since genetic tools for studying lethal mutations in Dicty are relatively poor, we did not proceed with generation of conditional mutants. We rather decided to biochemically delineate the functional implications of these proteins.

\subsection*{2.3.3 Cell-free assays with mycobacterial type I PKSs}

\subsubsection*{2.3.3.1 Cloning of disfp and diacps}

\textit{Disfp} gene has two exons with their sizes corresponding to 444 bp and 447 bp respectively for exons 1 and 2. The exons are separated by an intron of size 69 bp. Exons 1 and 2 were first PCR amplified from genomic DNA. Both the exons were then separately cloned into TOPO cloning vector (pCR666 and pCR667 respectively). The two exons then were joined together in TOPO vector to give pDN1 plasmid. Subsequently, the linked fragment was excised from the TOPO vector and inserted in pET-28a expression vector. All the clones were confirmed by restriction fragmentation pattern analysis, as indicated in Fig. 2.12. The fragments obtained matched with the expected fragmentation pattern as tabulated in Table 2.9.
Figure 2.12 Restriction fragmentation analysis of disf clone. Expected fragment sizes in bp are indicated in parentheses.

1. 100bp ladder
2. 1kb ladder
3. pCR666 BamHI, EcoRI (2580, 444)
4. 1kb ladder
5. 100bp ladder
6. pCR667 EcoRI, HindIII (2580, 447)
7. 100bp ladder
8. 1kb ladder
9. pCR666 NotI, EcoRI (2580, 444)
10. pCR667 NotI, HindIII (2580, 447)
11. 100bp ladder
12. 1kb ladder
13. pDN1 BamHI, HindIII (2580, 891)
14. 1kb ladder
15. 100bp ladder
16. pDN2 BamHI, HindIII (5369, 891)
17. 1kb ladder
18. 100bp ladder

<table>
<thead>
<tr>
<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 in TOPO</td>
<td>pCR666</td>
<td>BamHI, EcoRI (2580, 444)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NotI, EcoRI (2580, 444)</td>
</tr>
<tr>
<td>Exon 2 in TOPO</td>
<td>pCR667</td>
<td>EcoRI, HindIII (2580, 447)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NotI, HindIII (2580, 447)</td>
</tr>
<tr>
<td>disf full gene in TOPO</td>
<td>pDN1</td>
<td>BamHI, HindIII (2580, 891)</td>
</tr>
<tr>
<td>disf full gene in pET-28a</td>
<td>pDN2</td>
<td>BamHI, HindIII (5369, 891)</td>
</tr>
</tbody>
</table>

Table 2.9 Restriction digestion pattern of disf clone. Fragments sizes are in bp.
*Diacps* gene also has two exons of 68 bp and 432 bp respectively separated by a short exon of 94 bp. The two exons were amplified from genomic DNA using nested primers as described in Fig. 2.13. The two PCR products were mixed and used as template DNA in a second round of PCR, using the end primers. This step resulted in the amplification of whole gene which was cloned into TOPO cloning vector.

![Diagram](image)

*Figure 2.13 Amplification of complete *diacps* gene using nested primers.*

The 501 bp gene was then excised from the parent vector and cloned into pET-28a expression vector for protein expression (pRG25). As described earlier, all the clones

123
were confirmed with appropriate restriction enzymes as shown in Fig. 2.14. The fragmentation pattern has been listed in Table 2.10.

Figure 2.14 Restriction fragmentation analysis of *diacps* clone. Expected fragment sizes in bp are indicated in parentheses.

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>100bp ladder</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>1kb ladder</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>pCR561 <em>BamHI, HindIII</em> (2580, 501)</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2.10 Restriction digestion pattern of *diacps* clone. Fragments sizes are in bp.
2.3.3.2 Expression analysis and purification of DiSfp and DiAcps

*Disfp* and *diacps* clones were transformed into BL-21 expression strain of *E. coli*. Initial analysis for checking protein expression was performed on mini-scale by using 5 mL cultures at different temperature conditions (37°C, 30°C, 22°C, 18°C) and IPTG induction concentrations. The cells harbouring *Disfp* expression plasmid cultured at 37°C to an O.D.<sub>600nm</sub> of 0.6 and induced with 0.5 mM IPTG at 30°C for 8 hrs showed distinct induction of a protein. The SDS-PAGE mobility of this protein at ~34 kDa conformed to the expected molecular weight of *DiSfp* (Fig. 2.15). These conditions were used to set up batch cultures of 2 L and *his*-tagged *DiSfp* protein was purified to homogeneity from cleared cell lysate using affinity chromatography with Ni<sup>2+</sup>-NTA agarose to a concentration of 2-4 mg/mL (Fig. 2.15).

![SDS-PAGE Image](image.png)

**Figure 2.15 SDS-PAGE for Ni<sup>2+</sup>-NTA affinity purification of DiSfp.** Protein band at ~34 kDa represents *DiSfp* with *his*-tag.

1. Protein marker
2. Elution with 25 mM imidazole
3-8. Elution with 50 mM imidazole
9-14. Elution with 100 mM imidazole
BL-21 cells containing *diacps* expression plasmid were cultured at 37°C to an O.D._{600nm} of 0.6 and when induced with 0.5 mM IPTG at 18°C for 12 hrs, showed evident induction of a protein at around 27 kDa. The expected molecular weight of DiAcpS protein was determined to be around 20 kDa (taking into account *his*-tag as well). At higher induction temperatures also, the same band was seen to be induced, although a significant amount was observed to be going in the pellet rather than supernatant. This discrepancy in the expected and observed molecular weight was clarified using mass spectrometry-aided sequencing and this shall be discussed in the following section. To obtain higher protein yield, 2 L cultures were set up using the same conditions and purification was done using affinity chromatography (Fig. 2.16).

![SDS-PAGE](image)

**Figure 2.16 SDS-PAGE for Ni^{2+}-NTA affinity purification of DiAcpS.** Protein band at 27 kDa represents DiAcpS with *his*-tag.

1. Protein marker
2-5. Elution with 50 mM imidazole
6-10. Elution with 100 mM imidazole
2.3.3.3 **DiSfp and DiAcpS protein sequencing by mass spectrometry**

We performed peptide mass fingerprinting on the proteins to unambiguously establish their identities. In-gel trypsinization was carried out for both the purified proteins and the masses of peptides were identified by MALDI-MS. The MS and MS/MS data was then submitted to the web-based MASCOT search engine. MASCOT compares the experimental mass values with calculated peptide mass or fragmentation mass values obtained for proteins in various primary sequence databases (e.g. NCBI). The result thus generated includes a list of the matching proteins and parameters called 'score' and 'sequence coverage' that depict the significance of match. MASCOT search for pDN2 protein revealed *Dictostelium discoideum* DDB0186752 (gi 60466443) as the top match with a high confidence score of 287. Similarly, the 27 kDa band on MASCOT search identified *Dictostelium discoideum* DDB0217726 (gi 60471756) as the top hit. With both the proteins having been confirmed to be DiSfp and DiAcpS respectively (Fig. 2.17), we proceeded with the biochemical characterization of these enzymes.
Mascot Search Results

Protein View

Match to: gi|60466443 Score: 87 Expect: 0.0053
hypothetical protein DDB0186752 [Dictyostelium discoideum]

Nominal mass (M₀): 34331; Calculated pI value: 6.90
NCBI BLAST search of gi|60466443 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Dictyostelium discoideum

Variable modifications: Carbamidomethyl (C), Carboxymethyl (C), Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 53%

Matched peptides shown in Bold Red

1 MQNQESKLWN ILLQGDISK WKPDNEWEKQ INDFINDPIE SNRISHFKRF
51 NRWDWSLWK GRAMMELNVR RMLSLWYKSFKRTQHKSIF
101 YLSYTTTISN RFSNHIHDS NWVIGGSFL SDSIGIDIMD CKIPRHQIKMS
151 RFDPYMSSCF TDNNWKRING QKDDPSKIDL FPILWCLKES YIKADGKGLN
201 IELKSPFPII DQYKYGTAQPF ILMDDWENKK LLNNQFTFQF PYKRENKDDNN
251 NSFVIAICLD LTHNNHNNNN NNAULTNNIIT KDWSLQKLE SDFKIK

Mascot Search Results

Protein View

Match to: gi|60471756 Score: 236 Expect: 5.9e-018
hypothetical protein DDB0217726 [Dictyostelium discoideum]

Nominal mass (M₀): 18992; Calculated pI value: 5.94
NCBI BLAST search of gi|60471756 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Dictyostelium discoideum

Variable modifications: Carbamidomethyl (C), Carboxymethyl (C), Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 33%

Matched peptides shown in Bold Red

1 MRSKFGDAMD IVWISSERED KKEKHDQKFLK RAPWEVKEIR FKSLPNTASI
51 TESYDENNS ELNNINNINN INNINNNINRQ PEYLAGWQVA KESEYKAIGN
101 QDRSKLIFQDN IQIINESNMR PYVNLLELTE SYFKELGINK INLAISHDQTD
151 YAIKNVILES NESTKW

Figure 2.17 Confirmation of DiSfp (top) and DiAcpS (bottom) identity using mass spectrometry.
2.3.3.4 *DiSfp* and *DiAcpS* gel binding assays with mycobacterial type I PKSs

Enzymatic activity of the PPTases was first investigated with mycobacterial PKSs. Several of these PKSs have been previously cloned and characterized in our laboratory. Initial gel–binding assays were set up with mycobacterial PKS2 and [1-\(^{3}\)H] coenzyme A as substrates and the transfer of radiolabel was detected by autoradiography. A weak signal could be obtained only with DiSfp and not with DiAcpS (Fig. 2.18). We altered the assay conditions including, increasing the concentration of DiAcpS in the reaction mix but the profile remained unaltered.

![Figure 2.18 Gel-binding assay to check phosphopantetheinylation of DiSfp and DiAcpS on mycobacterial PKS2 using [1-\(^{3}\)H] CoA.](image)
Since PPTases are also known to use acyl CoA as substrates (Carreras et al., 1997; McAllister et al., 2000), we used [1-\textsuperscript{14}C] acetyl CoA for our further studies. Assays set up with three different multi-functional PKSs from Mtb - PKS2, PKS12 and MAS (mycocerosic acid synthase) with labeled acetyl CoA clearly indicated phosphopantetheinylation only by DiSfp and no activity could be observed for DiAcpS (Fig. 2.19).

![Diagram of Mycobacterial Type I PKS](image)

Figure 2.19 Gel-binding assay to check phosphopantetheinylation of DiSfp and DiAcpS on mycobacterial PKSs (as indicated) using [1-\textsuperscript{14}C] acetyl CoA. Only DiSfp is seen to be phosphopantetheinylating the type I mycobacterial PKSs.
We further probed if DiSfp was capable of transferring other acyl CoAs (hexanoyl and lauroyl CoA) on PKSs. Surprisingly, not only DiSfp but also DiAcpS showed ability to transfer both hexanoyl CoA and lauroyl CoA with comparable efficiencies (Fig. 2.20). This unusual ability to transfer longer chain acyl CoA by DiAcpS on type I PKS is perplexing and cannot be rationally justified at this stage of the work. We propose that this binding may be due to the binding of hydrophobic substrates in the active site hydrophobic groove of ACP.

![Figure 2.20 Extended specificity of DiSfp and DiAcpS with hexanoyl and lauroyl CoAs.](image)

Figure 2.20 Extended specificity of DiSfp and DiAcpS with hexanoyl and lauroyl CoAs.
Time-course study revealed maximal activity by DiSfp at 20 min. as is evident from Fig. 2.21 (a). Catalytic efficiency of DiSfp mediated phosphopantetheine transfer with respect to acetyl CoA was estimated by Michaelis-Menten fit and $k_{cat}/K_M$ was calculated to be 0.353 mM$^{-1}$ min$^{-1}$ (Fig. 2.21 (b)).

Figure 2.21 (a) Time-based kinetics of DiSfp. (b) Michaelis-Menten kinetic efficiency of DiSfp with respect to acetyl CoA.
2.3.4 *Cell-free assays with mycobacterial stand-alone ACP*

2.3.4.1 *Gel-binding assays*

To ascertain the activity of PPTases with type II ACP, mycobacterial stand-alone ACP (Rv1344) was used for experiments. The purified protein shows two bands on SDS-PAGE, a 13 kDa band corresponding to the intact protein size and an 11 kDa band. N-terminal sequencing of the lower band revealed it to be the truncated form of Rv1344, with 21 amino acids being absent from the N-terminus region. Gel binding assays with this stand-alone carrier protein showed a reverse trend of enzymatic activation. While DiAcpS could efficiently catalyze phosphopantetheinylation of Rv1344, DiSfp exhibited minor activity (Fig. 2.22). Surprisingly, DiSfp showed better activity with longer chain acyl CoAs (Fig. 2.23 (a)). Kinetics of DiAcpS with the ACP was determined by varying the concentrations of acetyl CoA and $k_{cat}/K_M$ estimated to be 0.162 mM$^{-1}$ min$^{-1}$ (Fig. 2.23 (b)).

Figure 2.22 Activity of Diety PPTases towards mycobacterial stand-alone ACP. Only DiAcpS phosphopantetheinylates Rv1344.
Figure 2.23 (a) Activity of Dicty PPTases towards mycobacterial stand-alone ACP with medium-chain acyl CoAs shows relaxed specificity. (b) Kinetics analysis of DiAcpS with respect to acetyl CoA.

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2.3.4.2 **HPLC-MALDI based assays**

In order to unambiguously confirm phosphopantetheinylation of ACP, these proteins were separated on reverse-phase column and subjected to mass spectrometric analysis. DiAcpS mediated conversion of apo- to holo-ACP resulted in shift in the retention time of the protein from 20.7 minutes to 19.4 minutes (Fig. 2.24 (a)). The peak at 19.4 minutes showed an increment of 340 Da, confirming modification with phosphopantetheine group. The enzymatic assays of apo-ACP (13,474 Da) with hexanoyl CoA and lauroyl CoA exhibited higher molecular masses of 13,913 Da and 14,003 Da respectively, consistent with attachment of corresponding acyl-phosphopantetheine group. Further analysis with DiSfp showed undoubtedly that it cannot efficiently catalyze conversion of apo-type II ACP to the holo-form, but can modify the apo-form with hexanoyl and lauroyl CoAs (Fig. 2.25 (a)). It is interesting to note that apart from the full-length protein, the truncated form of the carrier protein was also concomitantly modified by the PPTases, as is evident from the corresponding increase in the molecular mass. This indicates that deletion of ACP N-terminus has no effect on binding and transfer of p-pant arm by the PPTases.
Figure 2.24 (a) HPLC chromatogram of assays with DiAcPS. Reactions were carried out with CoA (peak 2), hexanoyl CoA (peak 3) and lauroyl CoA (peak 4). Peak 1 represents apo-form of ACP (b) MALDI-TOF analysis of HPLC peaks.
Figure 2.25 (a) HPLC chromatogram of assays with DiSfp. Reactions were carried out with CoA (no peak obtained), hexanoyl CoA (peak 3) and lauroyl CoA (peak 4). Peak 1 represents apoform of ACP. (b) MALDI-TOF analysis of HPLC peaks.
2.3.5 Activity with ACP fragment of mycobacterial PKS12

2.3.5.1 Cloning of PKS12 module 1 ACP

The differential specificity of DiAcps and DiSfps with large multi-functional PKSs and discrete ACP proteins prompted us to examine whether this specificity is due to architectural differences of the PKS protein or is an inherent property of ACP domain. To resolve this issue, we cloned and expressed the PKS12 ACP fragment and then studied its phosphopantetheinylation by the two PPTases. Boundaries of the module 1 ACP were defined using the PKS-NRPS database (Fig. 2.26).

Figure 2.26 Boundaries of PKS12 module 1 ACP identified using PKS-NRPS database.
The defined region was PCR amplified from pTC5 plasmid and cloned into pBS (pCR654). The fragment was then excised and cloned into pET-21c expression vector (pDN18). Clones were confirmed using restriction fragmentation analysis (Fig. 2.27 and Table 2.11).

![Restriction Fragmentation Analysis](image)

**Figure 2.27** Restriction fragmentation analysis of PKS12 module 1 ACP clone. Expected fragment sizes in bp are indicated in parentheses.

1. 1kb ladder
2. 100bp ladder
3. pCR654 Nhel, Xhol (2958, 255)
4. pDN18 Nhel, Xhol (5443, 255)
5. 100bp ladder
6. 1kb ladder

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<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
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<tr>
<td>PKS12 Module 1 ACP in pBS</td>
<td>pCR654</td>
<td>Nhel, Xhol 2958, 255</td>
</tr>
<tr>
<td>PKS12 Module 1 ACP in pET-21c</td>
<td>pDN18</td>
<td>Nhel, Xhol 5443, 255</td>
</tr>
</tbody>
</table>

**Table 2.11** Restriction digestion pattern of PKS12 module 1 ACP clone. Fragments sizes are in bp.
2.3.5.2 Expression analysis and purification of PKS12 module 1

**ACP**

pDN18 plasmid was transformed into BL-21 cells for expression of PKS12 ACP. Transformed cells were cultured at 30°C to an O.D.₆₀₀ₙₐₚ of 0.6 and induced with 0.5 mM IPTG at 22°C for 7-8 hrs. Protein was seen to be clearly induced and its molecular weight on SDS-PAGE corresponded to around 10 kDa which was consistent with the calculated molecular weight of PKS12 ACP (Fig. 2.28). Batch-purification from 2 L culture was performed and pure fractions of the protein were obtained using Ni²⁺-NTA chromatography. Protein yield was determined to be 3-5 mg/mL.

![Figure 2.28 SDS-PAGE for Ni²⁺-NTA affinity purification of PKS12 module 1 ACP. Protein band at ~10 kDa represents the ACP with his-tag.](image)

1. Protein marker
2-6. Elution with 50 mM imidazole
7-13. Elution with 100 mM imidazole
2.3.5.3  *PKS12 ACP protein sequencing by mass spectrometry*

We performed peptide mass fingerprinting on the purified protein to confirm its identity. In-gel trypsinization was carried out and the peptides were identified by MALDI-MS. The MS and MS/MS data was then submitted to the MASCOT search engine. MASCOT search for pDN18 protein showed mycobacterial PKS12 to be the top hit (gi 13881781). It can be observed in Fig. 2.29 that peptide fragments characterized by mass-spectrometry covered the entire region of module 1 ACP domain.
Mascot Search Results

Protein View

Match to: gi|13881781 Score: 458 Expect: 3.8e-040
polyketide synthase [Mycobacterium tuberculosis CDC1551]

Nominal mass (M): 431361; Calculated pI value: 5.07
NCEI BLAST search of gi|13881781 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Mycobacterium tuberculosis CDC1551
Links to retrieve other entries containing this sequence from NCEI Entrez:
  gi|15841536 from Mycobacterium tuberculosis CDC1551

Variable modifications: Carbamidomethyl (C), Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 6%

Matched peptides shown in Bold Red

1 MVDQLQHTAT ALRLALQVQEL REKHTNDALL EKSSPLAIAY GSRCPPCCGV
51 DSRPLSQWQAP ASDBWYSEPP TBGFDNLAG LPDFDPODVRH HSYARTGCVQ
101 DGQADPDRFAF PGISPESELA MDQPENMLE LEWALAEREP IDPTCRGSA
151 TCQVAGLQGV GCNMLAEHEII CYDLCGHTSS VASLRVAQVL GLEGRAVSD)
201 TACSSSIVAL HIAAVGSLQSG ECDLAALGTVV TVNATPTVFV EFSYHQLGLAP
251 DCEREPYQCCR AIGCVMSGEC CQLVQVLQSL ARBGLHVPVA UWWGAVQQD
301 GALSQFFOPN FSQQRVQMPA ALANAGLSAA EVVVGQVEGH GTTELQDPEIA
351 QAASSAYCQOD ROEGQPOPLNL GSQNNHSNEQ QAACAVONGI KMVLAMHSNKL
401 LPIAPIHLVQUP SVPHMVSAGA VELTAPFVW PACAPTDRAG VSSPGISCTMN
451 AHVLIIAVNSV VRRAAGAGAV PWVWVYWSAI SEASLRGQA QAAYAVQGDD
501 GLDVADVKCS LAGSRVHEHR AVVGVDRDR LLALDLNLAG DQLLGGSVVRG
551 TATAAKKTVF VPPQGQSQWL CMGELDLTAD PAPAQQIDRA AEAFAEFVDW
601 SLVWLGAPG GAPGDLPVRQ VQVPVLFAWNN SLAELWSVQA VHPDAVCHGS
651 GQGIIAAATVA GALSLRDAAR VTVLRSKLLA GLCPGFGWLS YACGADQARDB
701 LIAAAGGGLGS NQVNGSASV VUSOBBGAAE ELIATCSHRS LRTRRHIVDY
751 ASHSWEVEAA RPQNEALSGS ISPSTRTTVE FSSTUTGNLD TALGADVLYVY
801 RNQVRQTVLQD GAQVNEAECGG YRTFNEEESPH PALLTVETV PAACTDCDSDP
851 RSTVPTLRCG DQGIDLHFLLS AASAFVAGVA UVMQLYLDGA CVGELPTYAF
901 DKRVFVELSAR GSCADVSCGLG LGASHPLLLC AVVCLPAASC WLTCDLSY
951 VQPMUALPAPVD SEQULFDQTH QVPELAIGCD EVCSVSDLQ YLAPILLLA
1001 TGQVAYCQPVY IADQGNNHSD VSIFSDRDAQ AGWLILLASAI LPSOSVQPPAL
1051 DLSQWMPAAG VTVQVQFTYV QVPEVAQTVG PADQGAMPVW ADGCEFAEY
1101 RLFQAEAGCG CFCQDHALDD AVLHAHVATAC DPPDAPAPPA WQGSLHLATC
1151 ASMVEARRIAIP AGPSAIVSVEL ADQGLQVLVS VASMIARPVY HRLLLAASVC
1201 SCDQPLFRTV WPAAPAATSP GPTAQIFYER SVAADQDPVA GSYVSVHSQAL
1251 AAVQWULWTH ESQVLVTRAR GAMALPREDV ADLAGAVWGG LVRSAQFTHP
1301 GRIYVQGDDE ATDDAAIAAA LATGEQPGYSL AGQQTVargin RGMRADAIL
1351 VPPQGQSVWM GLSAGATPEN LLRLEVPVND APYGPFVQVW RBMEIAARFR
1401 DQIMTLMCHFT HIALLQDCEGA GVYQVQGCPV EFQVQSVQSF CFPQDOSGTL
1451 VAGQWRLLLP MPADYWAAR AASAVYFAAT YTAFAHLAVA YPGQVQLIHA
1501 GTGCQMAAV EAHRHCLELTV FATASKQGWDL TLRAQGFDSD HIDSRLSEIY
1551 EKDFRAATGCG EDFVVDLQD ALFEDDASL AEFLPGYFELR NEKTDIEFPQ

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Figure 2.29 PKS12 module 1 ACP sequencing by mass spectrometry.
2.3.5.4  **Enzymatic assays**

Gel-binding assays were set up with the purified protein and the two Dicty PPTases, as described earlier. It was clearly seen that the transfer of phosphopantetheine group on to the isolated PKS12 ACP domain could be catalyzed solely by DiSfp, concordant with what had earlier been observed with the ACP in integrated form (Fig. 2.30). This study unambiguously demonstrated that distinction between type I and type II ACP by the PPTases is guided by innate characteristics of the carrier protein, rather than in its modular organization.

![Figure 2.30](image)

*Figure 2.30 Autoradiography showing acetyl-phosphopantetheinylation of independent ACP domain of PKS12 by DiSfp.*
2.3.6 *PPTase specificity with Dictyostelium type I PKS/FAS*

2.3.6.1 *Enzymatic assays with ACP-type III PKS di-domain of DiPKS1*

The activity of Dicty PPTases was then evaluated by using cognate proteins from the organism. Since experiments with mycobacterial proteins suggested that modular arrangement does not determine the selectivity, we used a smaller di-domain protein to understand phosphopantetheinylation specificity. ACP-TypeIII PKS di-domain of DiPKS1 (Ghosh *et al.*, 2008) was used for the assays with both PPTases. Radiolabeling of the 66 kDa domain was observed only with DiSfp. Figure 2.31 illustrates that DiSfp is able to phosphopantetheinylate ACP-TypeIII PKS but DiAcpS fails to convert this *apo*-protein to its *holo* form. This reinforced our view that DiAcpS cannot function with type I PKS.

![Diagram of DiPKS1 and DiAcpS interaction](image)

Figure 2.31 Gel-binding assay with DiPKS1 ACP-typeIII PKS di-domain shows DiSfp to be specific for the type I DiPKS.
2.3.6.2  Cloning of ACP domain of DiPKS16

AcpS in several studies is suggested to be involved in primary metabolism of fatty acids (Gehring et al., 1997; Quadri et al., 1998; Mootz et al., 2001; Chalut et al., 2005). We therefore wanted to investigate if similar scenario exists in Dicty. Since DiPKS16 has been implicated as putative type I FAS (Zucko et al., 2007), we selected this protein for further analysis. However, presently there is no experimental evidence to existence of a FAS prototype in Dicty. DiPKS16 ACP domain boundaries were determined using the PKS-NRPS database (Fig. 2.32).

Figure 2.32 Boundaries of DiPKS16 ACP identified using PKS-NRPS database.
The 258 bp ACP region was PCR amplified from Dicty genomic DNA and cloned into pBS vector (pCR655) and then subsequently into pET-21c vector for protein expression (pDN19). Confirmatory results for clones are shown in Fig. 2.33. Fragmentation pattern has been tabulated in Table 2.12.

![Image of gel electrophoresis](image)

**Figure 2.33** Restriction fragmentation analysis of DiPKS16 ACP clone. Expected fragment sizes in bp are indicated in parentheses.

1. 1kb ladder
2. 100bp ladder
3. pCR655 *NheI, XhoI* (2958, 258)
4. pDN19 *NheI, XhoI* (5443, 258)
5. 100bp ladder
6. 1kb ladder

<table>
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<th>Clone description</th>
<th>Name of the clone</th>
<th>Restriction enzymes – Size of fragments generated (bp)</th>
</tr>
</thead>
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<tr>
<td>DiPKS16 ACP in pBS</td>
<td>pCR655</td>
<td><em>NheI, XhoI</em> 2958, 258</td>
</tr>
<tr>
<td>DiPKS16 ACP in pET-21c</td>
<td>pDN19</td>
<td><em>NheI, XhoI</em> 5443, 258</td>
</tr>
</tbody>
</table>

**Table 2.11** Restriction digestion pattern of DiPKS16 ACP clone. Fragments sizes are in bp.
2.3.6.3 **Expression analysis and purification of DiPKS16 ACP**

BL-21 cells harbouring pDN19 plasmid were cultured at 30°C to an O.D.\textsubscript{600nm} of 0.6 and induced with 0.5 mM IPTG at 22°C for 7-8 hrs. Protein was purified to homogeneity using affinity chromatography. The purified protein showed anomalous mobility on SDS-PAGE, indicating a molecular mass of ~27 kDa rather than the calculated mass of 10.6 kDa (Fig. 2.34). ACPs have been reported in literature to show abnormal migration on SDS-PAGE, owing to their highly acidic nature (Byers and Gong, 2007).

![SDS-PAGE for Ni\textsuperscript{2+}-NTA affinity purification of DiPKS16 ACP. Protein band at 27 kDa represents the ACP with his-tag.](image)

1. Protein marker
2-5. Elution with 25 mM imidazole
6-8. Elution with 50 mM imidazole
9-10. Elution with 100 mM imidazole

2.3.6.4 **DiPKS16 ACP protein sequencing by mass spectrometry**

Protein band on SDS-PAGE was excised and subjected to tryptic digestion for sequencing on MALDI. MASCOT search predicted the protein to be DiPKS16 ACP (Fig. 2.35).
Mascot Search Results

Protein View

Match to: gi|12082983|Score: 429 Expect: 3e-037
similar to Anabaena sp. (strain FCC 1120). Polyamide synthase [Dictyostelium discoideum]

Nominal mass (M): 239724; Calculated pI value: 5.76
NCBI BLAST search of gi|12082983| against nr
Unformatted sequence string for pasting into other applications

Taxonomy: Dictyostelium discoideum
Links to retrieve other entries containing this sequence from NCBI Entrez:
|gi|16047181| from Dictyostelium discoideum

Variable modifications: Carbamidomethyl (C), Carbamethylation (M)
Cleavage by Trypsin: cons C-term side of KA unless next residue is P
Sequence Coverage: 9%

Hatched peptides shown in Bold Red

1 MTFAWVDFEN MVEAQICIQCG FAPPQVQVR WDFVQDNAE AFIVKGDQK
5 KXKERVGGVTQ YNNQYQGTV TCSQWNNDDP LFFQSTSDPE PTDGDQQEML
101 HTYDLRAFED AAIKPSKQTV SGTVFQYEM WLVQYQOFQR ESLTIPVTIV
151 TCSAGSPQEGG KEPSQFPQG PGSTLQVAC SGEVP次数 QAIAGQGKMK
201 AIVQUGNCLG DSQSHNFTSG LQMLCHQGQC RSSARSCGF RSEEGGCVCI
251 LKVDVYDNDK GSTRTYVING GSVRYDDQNA KTNIQFSMK AQGSEKETL
301 EWGQGQDSEQ YTVKATICGT YQCRPHIKIA YSEYQPDHMT QPSLTVQGV
351 KRSNKHLSGA AMGIALKAVL SLSHSLVLD HNVFPHNPQK KPEDWQVIR
401 TVDQFQSKTV KLYNMGYESC GLSSNQCGNL LERAPINFR LKKTNNSST
451 SSSSDDOQK YLIPFQANIN ISKSTVVEVLN ISRQITVSTL LFQFQKYNK
501 TEYQXNLDKR YKATAFXDE FLNTHMTIS TSSSSTSAIA RASTPVYTVY
551 PFQGQGQGER MQALYGLKE UQEAIDGCDO KLLATYGFQ LQGKLEQKLS
601 DSPEKHFHPD LAQSFQHQLGG VLQVLKTHSK GTSPYTVQVG KGQWVHSLF
651 SQVSVLSFHK KTVVTQLAG MLHSTGQRL SICGADVAST RCKALFQRP
701 EIACTQKPIY TIVTQQGOL LCAQSTGIS QYCAFLQTF GPQHNSQQKM
751 IHEKCIADLL DLQVPNVFTQP FFPSSTTOSG LSHDFYVWQ YTMNLFPMV
801 EFTRAIQNET NFIRKNEENYW NAIFLEQRFQ PTLPYQIFIR EPSNFSSTYK
851 PEYKLPLRNK KERLTFQPLA LSTLQVHYQ IDPASGGQQL LTTSSAGGGD
901 IDPQWEENK LPKQGDQFR YMDQTRPEG NVKSPSNFLN QDRGAFNTL
951 KNFVDFQPSL HOQYVGCGC HNLWPKQGD QYDMLQKQFQ QQDITINFL
1001 FSNNPFLQFSG WYRQTFSTP PTTKSVQDVE FFIDFSMNQ SFVQTSTCHIK
1051 GLFQKFPANQ KRLISQGKIS CSTSMULSY DRNLLQLAL PVGGFQPGES
1101 SCISGGCSYS FKLQMSFCHE FOMDFLQPS ICAGHYLVQ LSEQGFQVFF
1151 DLQGMCPTY WMQSFVQFQ ITFAPFQCF VGNSNHSSL NLQMODGLLL
1201 SGNQYXKSTD ILETQYKQFQ PGSTQNYGM QGDSPSPTLE KIELKWLKRS
1251 NKINAPPQIK NEMNYLQMDL QNKDFQMDL QFQVDKSTL WTDLSEDSSN
1301 TKLNKKEQSQ NTTIDSRQK QBLASLQAV XEKSSFRKQ XNLBSNKR
1351 IVSLLQGKDS HNFSPQFSG PRTQTFQNS NCSNYSNNT SGADDYHNR
1401 ETFTQANSP FSHFPQFQG FQHPQISLPW UNRDOLOLNS YRGECHLKI
1451 IIDSRSTQNP QNLSKLLQLQ QGLMLLMSN NNNRSTTTI PDSQNEIDIS
1501 KEHNSAQNL AKIRYSDFQ QDQSLNSYQ NNDLQILT LLYQVEINSID
1551 SNFQVLKSYK LLLPDQGIQK NFQPDVLSF NLLKANDQKQ KLLIKQKQI
1601 KSLIRYCFSTK LQTTNINQDO DBEQQQOPS ILIVOTEQDP KESMSFIVSS
2.3.6.5  Gel binding assays with DiPKS16 ACP

Phosphopantetheinylation of this ACP was followed by autoradiography with radiolabeled acetyl CoA. Yet again labeling of the protein could be detected only when DiSfp was used in the reaction mix (Fig. 2.36). In contrast, the lane with DiAcpS showed no labeling at all.

Figure 2.35 Mass-spectrometric confirmation of DiPKS16 ACP.

Figure 2.36 Phosphopantetheinylation of DiPKS16 ACP mediated solely by DiSfp and not by DiAcpS.
2.3.6.6 **HPLC-MALDI based assays with DiPKS16 ACP**

HPLC-MALDI-TOF based assays were performed to provide unambiguous proof to the selectivity of the Dicty PPTases towards DiPKS16 ACP. As is evident from Fig. 2.37(a), DiSfp resulted in a shift of ACP retention time on reverse-phase column from 20.5 minutes to 19.2 minutes. These peaks on MALDI-TOF revealed molecular masses of 10,599 Da and 10,938 Da respectively (Fig. 2.37(b)). The increment of 340 Da is consistent with attachment of a phosphopantetheine arm. Similar activity was also observed with hexanoyl and lauroyl CoA, with expected increments in the molecular weight of ACP. ACP modified with hexanoyl CoA showed a retention time of 19.7 minutes and molecular mass of 11036 Da. Lauroyl CoA treated protein eluted at 20.1 minutes and revealed a molecular mass of 11124 Da. As opposed to this, DiAcpS lacked any activity towards this ACP. Intriguingly, DiAcpS failed to modify the type I ACP even with hexanoyl and lauroyl CoA. This is in contrast to our earlier observation with mycobacterial PKSs.
2.3.7 **PPTase specificity with Dictyostelium stand-alone ACP**

2.3.7.1 **Identification and cloning of DiACP**

Our data strongly suggests that all multi-functional PKSs are modified by DiSfp. This however raised an issue on the essential requirement of DiAcpS that was reflected in our genetic studies. A BLAST search by using *Saccharomyces* stand-alone ACP with Dictybase identified DDB0238774 as a putative type II ACP, annotated as component of NADPH ubiquinone reductase (Fig. 2.38). The two sequences exhibited a high similarity at 65%. No other homologs of this protein were found in the Dicty genome. In the subsequent sections, the gene would be referred to as *diacp*. 

Figure 2.37 (a) HPLC chromatogram of assays with DiSfp and DiPKS16 ACP. Reactions were carried out with CoA (peak 2), hexanoyl CoA (peak 3) and lauroyl CoA (peak 4). Peak 1 represents apo-form of ACP (b) MALDI-TOF analysis of HPLC peaks.
Figure 2.38 BLAST search on Dictybase against Saccharomyces stand-alone ACP.

The 363 bp gene was amplified from Dicty genomic DNA and inserted into TA vector (pCR423). Following this, the fragment was removed and cloned into pET-21c and the clone was named pDN15. Analysis of clones was done using appropriate restriction enzymes (Fig. 2.39 and Table 2.12).
Figure 2.39 Restriction fragmentation analysis of *dia*cp clone. Expected fragment sizes in bp are indicated in parentheses.

1. 1kb ladder
2. 100bp ladder
3. pCR423 *NheI, XhoI* (3015, 363)
4. pDN15 *NheI, XhoI* (5443, 363)
5. 1kb ladder
6. 100bp ladder

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<th>Clone description</th>
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<th>Restriction enzymes – Size of fragments generated (bp)</th>
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<td>pCR423</td>
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<td><em>DiACP</em> in pET-21c</td>
<td>pDN15</td>
<td><em>NheI, XhoI</em> 5443, 363</td>
</tr>
</tbody>
</table>

Table 2.12 Restriction digestion pattern of DiACP clone. Fragments sizes are in bp.

2.3.7.2 *Expression analysis and purification of DiACP*

Preliminary investigation on optimum conditions for the expression of this protein was performed with 5 mL cultures. pDN15 clone was transformed into BL-21 cells and the cells were initially grown at 30°C till O.D. reached 0.6. This culture was then induced with 0.5 mM IPTG at 18°C. Protein was seen to be prominently induced under this set of conditions. However, it showed a mobility of ~10 kDa on SDS-PAGE, less than the expected molecular weight of 13 kDa (Fig. 2.40). We proceeded
with identification of the protein on MALDI after Ni$^{2+}$-NTA chromatographic purification from batch cultures.

![SDS-PAGE](image)

*Figure 2.40 SDS-PAGE for Ni$^{2+}$-NTA affinity purification of DiACP. Protein band at ~10 kDa represents the ACP with his-tag.*

1. Protein marker
2-5. Elution with 25 mM imidazole
6-11. Elution with 50 mM imidazole

### 2.3.7.3 DiACP protein sequencing by mass spectrometry

Gel extraction of trypsinized fragments on analysis on MALDI-TOF confirmed the identity of protein (Fig. 2.41); however peptides from N-terminus could not be detected. HPLC-purified intact protein analysis with MALDI-TOF showed the molecular weight of this protein as 10109 Da. This deviation from the predicted molecular weight (13195 Da) could be due to N-terminus truncation of the protein and can be accounted by loss of 24 amino acids. Interestingly, sequence analysis based on MitoProt software predicted a 0.99 probability of the protein being localized in mitochondria (Fig. 2.42). It is possible that this N-terminus could in fact be a signal for mitochondrial import.
Mascot Search Results

Protein View

Match to: gi|60463271 Score: 80 Expect: 0.023
*hypothetical protein DDB0184099 [Dictyostelium discoideum]*

Nominal mass (Mw): 13188; Calculated pI value: 4.99
NCBI BLAST search of gi|60463271 against nr
Unformatted sequence string for pasting into other applications

Taxonomy: *Dictyostelium discoideum*

Variable modifications: Carbamidomethyl (C), Carboxymethyl (C), Oxidation (M)
Cleavage by Trypsin: cuts C-term side of KR unless next residue is P
Sequence Coverage: 53%

Matched peptides shown in Bold Red

<table>
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<th>Peptide</th>
<th>Matched</th>
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<tr>
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<tr>
<td>DRRSCBSTV SYLIRKFTAK</td>
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Figure 2.41 Identification of DiACP using mass-spectrometry.
MitoProt II - v1.101

Input sequence length : 120 aa

VALUES OF COMPUTED PARAMETERS

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HYDROPHOBIC SCALE USED

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PROBABILITY

of export to mitochondria: 0.9909

Figure 2.42 Analysis of DiACP sequence with MitoProt software predicts it to be mitochondrially localized.
2.3.7.4 **Gel-based assays**

Similar to earlier studies with mycobacterial type II ACP, *Dictyostelium* stand-alone ACP was seen to be radiolabeled on gel only by DiACP S and not by DiSfp (Fig. 2.43). Thus, it could be asserted based on these results that phosphopantetheinylation activity of DiACP S is specific to type II ACPs.

![Diagram](image)

**Figure 2.43** Phosphopantetheinylation of DiACP by DiACP S; no activity is observed with DiSfp.
2.3.7.5  **HPLC-MALDI based assays**

To unambiguously confirm the phosphopantetheinylation by DiAcpS, modified protein was subjected to HPLC and the eluted protein was analyzed on MALDI-TOF. The protein peak was obtained at a retention time of 19.7 minutes as against 20.7 minutes of *apo*-ACP, and showed an increase in molecular mass from 10109 Da to 10449 Da (Fig. 2.44 (a)). Similar reaction with hexanoyl CoA also led to a shift in the retention time to 20.4 minutes. As expected, HPLC peak analysis on MALDI showed an increase of 441 Da in the molecular mass of the protein (Fig. 2.44 (b)).

![HPLC chromatogram](image)

**Figure 2.44** (a) HPLC chromatogram of assays with DiAcpS and DiACP. Reactions were carried out with CoA (peak 2) and hexanoyl CoA (peak 3). Peak 1 represents *apo*-form of ACP (b) MALDI-TOF analysis of HPLC peaks.
Overall, our studies indicate that DiSfp is specific to type I PKS and FAS. DiAcpS on the other hand, shows selectivity towards type II ACP.

2.4 DISCUSSION

In this chapter we report identification and characterization of two phosphopantetheinyl transferases – DiAcpS and DiSfp that show distinctive ability to post-translationally modify architecturally different proteins. While function of DiAcpS is restricted to activation of small ACP proteins, probably involved in cell respiration, Disfp is shown to convert apo-multifunctional PKS/FAS proteins to their holo-forms.

In the present study, we have resolved the recognition ability of these PPTases by using several intact multifunctional PKSs as well as mono- and di-domain protein fragments. In all the instances investigated in this study, the smaller domain fragments of type I multifunctional proteins retained their specificity to be modified by DiSfp and DiAcpS did not show activity with these engineered domains. Clearly, our work shows that the specificity of phosphopantetheinylation is not dictated by the modular architecture, but in fact involves specific recognition of the carrier domains. An earlier study with rat FAS ACP domain however, had come to reverse conclusion (Reed et al., 2003). In that case an independently expressed ACP domain from multifunctional FAS system could be modified by bacterial AcpS that is known to modify Type II FAS ACP proteins. This apparent contradiction could be a manifestation of a broader specificity of AcpS protein or this could also be argued in
terms of the projected evolutionary relationship of two PPTases with primary and secondary metabolism (Mootz et al., 2001; Chalut et al., 2005; Lambalot et al., 1996; Gehring et al., 1997). It must be emphasized that the FAS biosynthetic system from Dicty has not been characterized. Based on bioinformatics analysis, DiPKS16 and DiPKS17 were suggested to be putative FASs and we have therefore examined the specificity of DiSfp and DiAcpS with ACP domain of DiPKS16. Here, ACP domain fragment showed strict specificity for its modification by DiSfp. This data coupled with a similar observation in case of DiPKS1 di-domain fragment and ACP domain fragment of mycobacterial PKS12, strongly indicates that DiSfp specifically recognizes type I ACPs.

The inability of DiAcpS to phosphopantetheinylate type I assembly line prompted us to search for type II ACP in Dicty. Although ACP sequences could be fairly divergent, a careful analysis of the genome using NRPS-PKS database provided DDB0238774 (old annotation - DDB0184099) as the only significant hit. Notably, this ACP has been annotated as NADH dehydrogenase ubiquinone 1 in the Dictybase suggesting it to be a part of the mitochondrial respiratory chain. Dictyostelium stand-alone ACP (DiACP) clearly showed homology with the yeast ACP, which possesses N-terminal leader sequence of about 35 amino acids that is proposed to be toxic for its expression in E. coli (Stuible et al., 1998). Interestingly, expression of complete diacp gene in E. coli yielded truncated protein with deletion at the N-terminus. In Saccharomyces, mtACP is instead known to be involved in octanoate biosynthesis which is a precursor to lipoic acid (Brody et al., 1997). MtACP in Neurospora crassa
has been demonstrated to be essential for the structural integrity of complex I of respiratory chain (Sackmann et al., 1991).

The recognition and specificity of PPTases at this stage cannot be unambiguously defined based on sequence homology and the three-dimensional structural analyses along with mutagenic studies are providing insights into this process (Reuter et al., 1999; Parris et al., 2000; Chirgadze et al., 2000, Mofid et al., 2004; Finking et al., 2004). Structural studies show that the active site of Sfp is shallower and wider as compared to that of AcpS. It has therefore been reasoned that type I carrier protein, being part of a larger multifunctional protein, can fit into the substrate pocket of Sfp. Type II ACP on the other hand, is suggested to suitably adapt both into the deep and narrow active site of AcpS and the shallower Sfp active site (Parris et al., 2000). Our results however show that DiAcpS and DiSfp exhibit stringent selectivity towards type II and type I ACPs respectively. Remarkably, this specificity was relaxed when longer chain acyl CoAs were used for mycobacterial proteins. In case of Dicty ACPs, PPTases adhered to their selectivity criterion even with the other acyl CoAs. This observation underscores the differences in the carrier proteins of the two organisms. Since mycobacterial PKSs are involved in biosynthesis of complex lipids which utilize long-chain lipids, it is possible that mere binding in the active site pocket could initiate this transfer of acyl phosphopantetheine chain.
Analysis of transcriptional profile of *Dictyostelium* PKSs by real time PCR has shown that the different PKSs are expressed at distinct developmental stages of the organism (Zucko *et al.*, 2007). This makes it mandatory for the appropriate PPTases to be also present at all these stages. Our best efforts to obtain genetic knockouts of these genes were unsuccessful and provided only non-homologous recombinants. In an earlier study with an essential *phlp2* gene in Dicty, slow-growing colonies (which also showed early death) in the initial phase of screening were shown to contain homologous recombination (Blaauw *et al.*, 2003). However, we did not observe any such trend with the PPTase knockouts. A careful investigation of the expression of genes by RT-PCR showed presence of both the PPTase during all the stages of Dicty life cycle. These evidences suggest the essential nature of DiAcpS and DiSfp for the amoeba.

In conclusion, we have shown clear demarcation in the functional roles of two PPTases in *Dictyostelium*. Through their action on PKS/FAS systems, these enzymes are expected to be vital in the initiation and progress of developmental pathway.