Streptomyces coelicolor phosphopantetheinyl transferase: a promiscuous activator of polyketide and fatty acid synthase acyl carrier proteins

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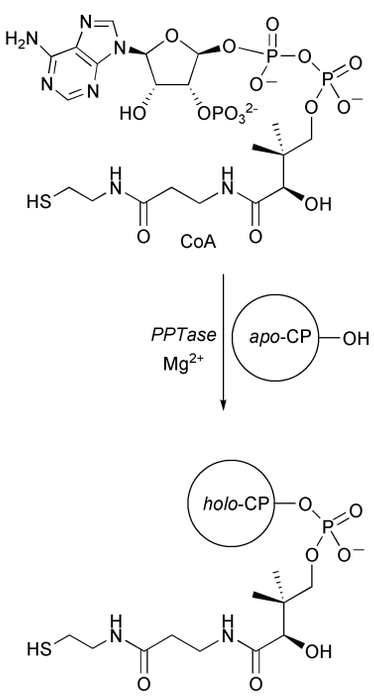
Streptomyces coelicolor is host to a number of biosynthetic proteins requiring post-translational modification by the addition of phosphopantetheine groups. The *S. coelicolor* genome, was probed, *in silico*, with the sequence of *Escherichia coli holo*-Acyl Carrier Protein Synthase (ACPS). A single open reading frame (ORF) strongly matching the *E. coli* ACPS was discovered. The putative *S. coelicolor* ACPS ORF was cloned and expressed and the resulting protein purified and characterised. *S. coelicolor* ACPS appears to be extremely promiscuous in its substrate specificity, accepting varied acyl CoA substrates and protein substrates from Type I and Type II fatty acid synthases (FAS) as well as from Type I and Type II polyketide synthase (PKS) biosynthetic protein complexes. This phosphopantetheinyl transferase thus has high potential for the synthesis of diverse *holo*- and acylated acyl carrier proteins.

Introduction

The biosynthesis of numerous important primary and secondary metabolites takes place on large protein synthase complexes in bacteria, fungi and plants. In general small building blocks, derived from primary metabolism, are localised and extended on these protein scaffolds.[1] In this way, fatty acids and polyketides are assembled from simple carboxylic acids and non-ribosomal peptides are assembled from amino acids. Except for the chalcone, stilbene and related Type III synthases, all these biosynthetic protein complexes use specialised carrier proteins (CP) to hold the assembly intermediates during biosynthesis. These partially complete metabolites are attached *via* thiol esters to the terminal thiol of a phosphopantetheine (PP) prosthetic group which is covalently attached to the CP. For this reason, synthase *apo*-CP components (lacking PP) are inactive.[2]

Carrier protein PP groups are derived from CoA, *in vivo*, and the transfer of PP from CoA to the *apo*-CP is catalysed by phosphopantetheinyl transferase (PPTase) enzymes (Scheme 1). These can be either isolated proteins or defined domains of multifunctional modular proteins. The PP is attached to the hydroxy of a conserved serine residue of the CP.

Streptomyces coelicolor is a Gram positive bacterium known to produce the polyketide antibiotic actinorhodin **1**, the cyclopentanone antibiotic methylenomycin A **2**,[3] the immunosuppressive alkaloid undecylprodigiosin **3**[4] and the non-ribosomal peptide antibiotic CDA **4**.[5–7] The genome of *S. coelicolor* also harbours biosynthetic genes encoding other synthases such as *WhiE*, which manufactures many compounds including the dodecaketide **5** when expressed in heterologous hosts.[8] In addition to these compounds, *S. coelicolor* produces the usual range of bacterial fatty acids. *S. coelicolor* is also often used for the *in vivo* expression of synthase components derived from other organisms. These include discrete PKS components, such as bacterial acyl carrier proteins (ACPs), as well complete synthases such as those responsible for 6-methylsalicylic acid **6** and 6-deoxyerythronolide B **7** biosynthesis.[9–11]



Scheme 1 Conversion of *apo*-CP to *holo*-CP.

All of these compounds require active *holo*-CPs for biosynthesis. The gene cluster encoding undecylprodigiosin **3** biosynthesis does appear to contain a PPTase gene,[4] but other biosynthetic gene clusters in *S. coelicolor* do not. It seemed likely, therefore, that *S. coelicolor* contains a multifunctional PPTase capable of activating a wide range of CPs. With the advent of the completed *S. coelicolor* genome sequence we attempted to locate and clone this putative *S. coelicolor* PPTase and study its activity. An understanding of the substrate

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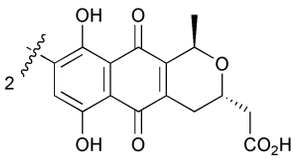
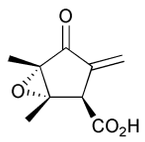
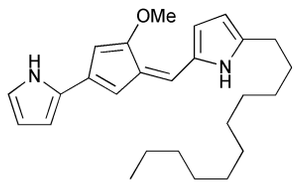


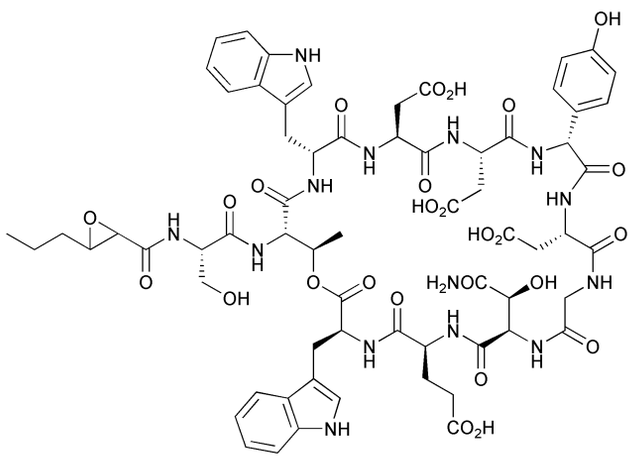
1 Actinorhodin



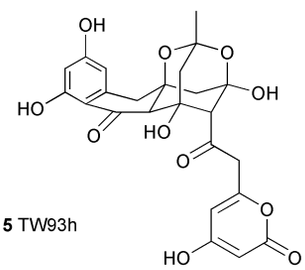
2 Methylenomycin



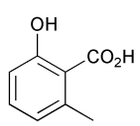
3 Undecylprodigiosin



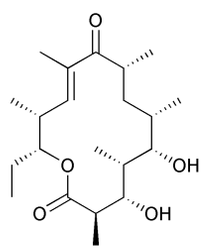
4 Calcium dependent antibiotic (CDA)



5 TW93h



6 6-Methylsalicylic acid



7 6-Deoxyerythronolide B

specificity of *S. coelicolor* PPTases will be important for future metabolic engineering of this organism for the *in vivo* synthesis of new compounds.

Results

Marahiel has identified a protein superfamily of PPTases which includes enzymes which transfer PP to acyl carrier proteins (ACP) from fatty acid synthases (FAS) and polyketide synthases (PKS) – these proteins are known as *holo*-ACP synthases (ACPS).[12] The PPTase superfamily also includes enzymes which transfer PP to the peptidyl carrier protein (PCP) moieties of non-ribosomal peptide synthases (NRPS).[12] We have previously used the *E. coli* ACPS[13] in co-expression experiments with Type II PKS ACPs in *E. coli* and shown that it is active with a variety of PKS ACPs *in vivo*. However, Walsh has shown that *E. coli* ACPS does not have a very broad substrate specificity *in vitro*.[14] For this reason we wished to access a much more catholic enzyme and for the reasons discussed above we believed that the *S. coelicolor* ACPS could be suitable.

The known *E. coli* ACPS DNA sequence was used to search the *S. coelicolor* genome. Pleasingly a putative open reading frame (ORF) with strong homology to *E. coli* ACPS was detected on cosmid SC6G4.[15] No other close matches were found. Pile-up and tree-plot analysis (Fig. 1) revealed close protein sequence homology to *E. coli* ACPS as expected (33% identity, 45% similarity of the translated polypeptide), but also similarity to various fungal FAS PPTases and bacterial NRPS PPTases.

We designed polymerase chain reaction (PCR) primers matching the 3′ and 5′ terminal sequences of SC6G4/ACPS. PCR from *S. coelicolor* genomic DNA then gave a synthetic DNA product of the expected *ca*. 400 base pairs (bp) (Fig. 2A) which was cloned and sequenced. Sequencing confirmed the identity of the product which was then sub-cloned into pET15b. This vector encodes an *N*-terminal fusion consisting of a hexahistidine (his_6) sequence, followed by a thrombin (protease) cleavage site.

Expression in the usual way yielded *S. coelicolor* ACPS with an *N*-terminal his_6 tag. The expressed protein was purified by Ni^{2+} and ion exchange chromatography (Fig. 2B). ESMS analysis of the purified protein was carried out (Fig. 3A). Transformation of the raw spectrum indicated that one, two or three Ni^{2+} ions remained attached to the protein (Fig. 3B). Treatment with the protease thrombin gave a *ca*. 50 : 50 mixture of proteins with, and without, the his_6 tag. Further treatment with thrombin did not improve this ratio. However, the partially cleaved protein was no more active than his_6-ACPS (*vide infra*) and so the his_6-protein was used in all further experiments. Calibrated gel filtration chromatography revealed the protein to be predominantly trimeric (measured 40 000 Da, calculated 43 794 Da). The *E. coli* ACPS has been reported to behave as a dimer under similar chromatographic conditions.[14] This is in contrast with the recent results of Parris and Somers who showed that the *Bacillus subtilis* ACPS exists as a trimer in solution when complexed with ACP,[16] while Chirgadze has also shown that the ACPS from *Streptococcus pneumoniae* is trimeric.[17]

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E. coli ACPS    .AILGLGTDI VEIARIEAVI ARSGDRLARR VLSDNEWAIW
S. coelicolor   MSIIGVGIDV AEVERFGAAL ERTPALAGRL CFLESELLLP

E. coli ACPS    KTHHQPVRFL AKRFAVKEAA AKAFGTGIRN GLAFNQFEVF
S. coelicolor   GGERRGVASL AARFAAKEAL AKALGAPAGL LWTDAEVWVE

E. coli ACPS    NDELGKPRLR LWGEALKLAE KLGVANMHVT LADERHYACA
S. coelicolor   AG..GRPRLR VTGTVAARAA ELGVASWHVS LSHDAGIASA

E. coli ACPS    TVIIES
S. coelicolor   VVIAEG
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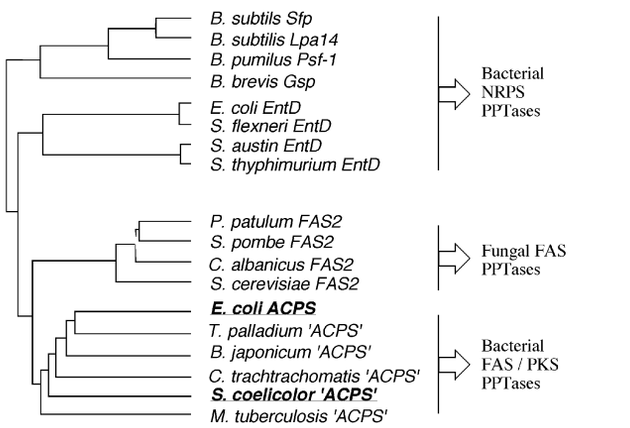


Fig. 1 Sequence comparison of *S. coelicolor* ACPS with other PPTases.

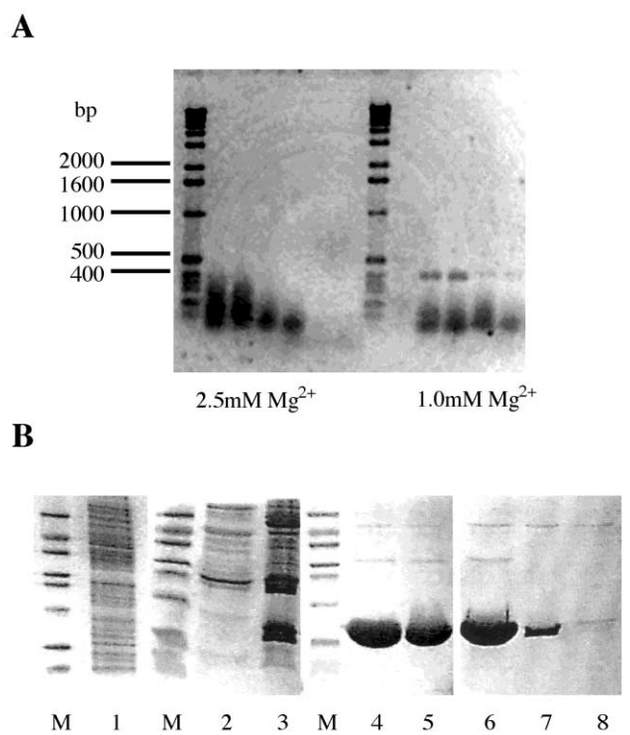


Fig. 2 PCR product obtained from *S. coelicolor* genomic DNA template. **B**: Expression and purification of *S. coelicolor* ACPS from *E. coli*. Lanes: 1, protein supernatant after Ni^{2+} binding; 2–3, eluted wash buffer; 4–8, imidazole elution; **M**, molecular weight markers, from top 66 kDa, 45 kDa, 36 kDa, 29 kDa, 24 kDa, 20 kDa, 14.2 kDa, 6.5 kDa.

Two likely substrates of *S. coelicolor* ACPS are the FAS ACP and the ACP involved in actinorhodin (*act*) biosynthesis. Thus FAS *apo*-ACP and *act apo*-ACP from *S. coelicolor* were prepared and purified from over-expressing strains of *E. coli* according to published methods.[18,19] These proteins were incubated with the purified ACPS, CoA and Mg^{2+} for 2 h. After this time the protein components were extracted from the assay mixture by the addition of butylated silica particles. Centrifugation separated the solid matrix, which was washed and then the bound proteins were eluted with CH_3CN. The

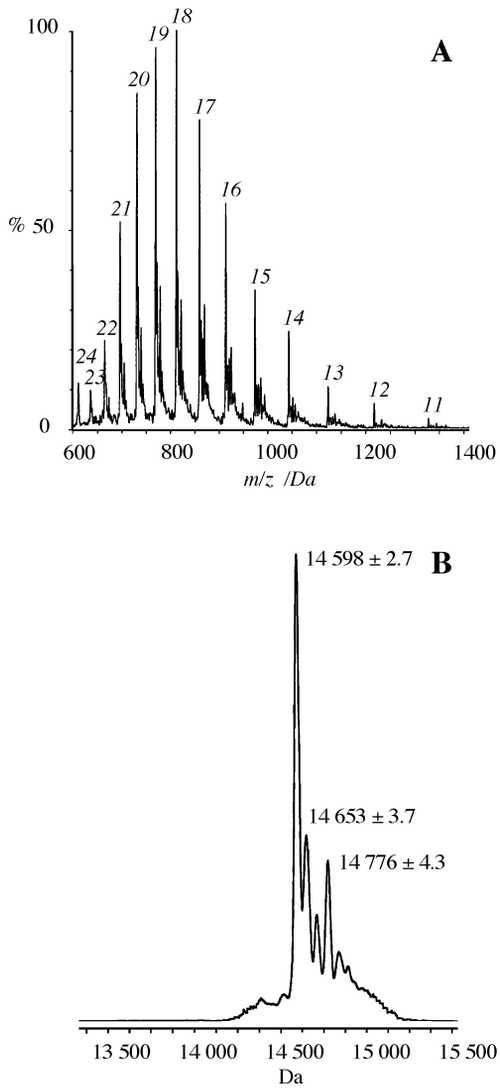


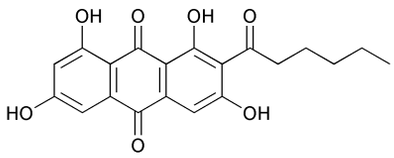
Fig. 3 ESMS analysis of *S. coelicolor* ACPS. **A**: raw MS data showing mass charge states. **B**: transform data. Calculated masses: his_6-ACPS (minus *N*-terminal methionine) 14598.6; his_6-ACPS (minus *N*-terminal methionine plus Ni^{2+}) 14656.6; his_6-ACPS (minus *N*-terminal methionine + $2Ni^{2+}$) 14714.6; his_6-ACPS (minus *N*-terminal methionine + $3Ni^{2+}$) 14772.6.

extracted proteins were then examined by ESMS. The purified ACPS clearly converted the *apo*-forms of both ACPs to their *holo*-counterparts (Table 1). In both cases full conversion to *holo*-ACP was observed in 2 h. In the absence of ACPS, no PP transfer was observed.

We then examined the activity of *S. coelicolor* ACPS with heterologous ACPs. *E. coli* FAS *holo*-ACP was obtained (Sigma) and the PP linkage hydrolysed by treatment with HF[20] followed by repurification. In addition, the Type II PKS ACP involved in oxytetracycline (otc) biosynthesis in *Streptomyces rimosus* was obtained using published procedures.[18,21,22] Once again both *apo*-ACPs were substrates for the *S. coelicolor* ACPS (Table 1).

The ACPs examined so-far are from Type II systems where several discrete proteins form a synthase complex and the ACP is a relatively small component. This differs from Type I systems which are large multifunctional proteins in which the ACP is an embedded domain. In order to test whether the *S. coelicolor* ACPS could catalyse transfer of PP to Type I synthases, the embedded ACP domains from a mammalian FAS (rat FAS)[23] and from a fungal PKS (norsolorinic acid **8** synthase from *Aspergillus parasiticus*) were cloned and expressed as individual proteins. The *apo*-ACPs were obtained as before and incubated with the *S. coelicolor* ACPS. Once again these

proteins proved to be substrates for *S. coelicolor* ACPS (Table 1) and were fully converted to their respective *holo*-forms.



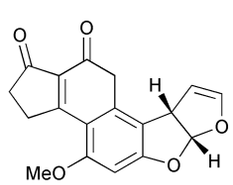
8 Norsolorinic acid

E. coli ACPS is capable of catalysing the transfer of acyl-PPs from acyl-CoAs to *apo*-ACPs and it is also capable of transferring 'unnatural' CoA analogues to give the corresponding acylated ACPs.[14] We thus tested the ability of *S. coelicolor* ACPS to use a range of commercially available CoA derivatives as substrates. Using the same assay system, we used acetyl, malonyl, methylmalonyl, propionyl, *n*-butyryl, isobutyryl, acetoacetyl, hexanoyl and benzoyl CoAs. In all cases examined, the acyl CoA was transferred to the *apo*-ACP to form the corresponding acyl-ACPs (Table 1).

Discussion

S. coelicolor ACPS was cloned and expressed after rapid identification of its encoding gene by electronic searching. The purified protein transfers PP from CoA to the conserved serine of both endogenous Type II ACPs from *S. coelicolor* and to heterologous Type II ACPs from other bacteria *in vitro*. ACPS is also active towards the ACP domains from a Type I mammalian FAS and a Type I fungal PKS. Although these Type I substrates show some sequence homology to the Type II ACPs and possess the conserved serine for PP attachment, it was not clear at the outset of this study that they would be sufficiently similar to act as substrates. These results show that the Type I ACP domains must be substantially similar to their Type II counterparts. Furthermore, even though they are expressed out of context, away from the other Type I protein domains, it appears that these proteins must be properly folded.

In addition to studying the protein selectivity of *S. coelicolor* ACPS we also examined whether, like other ACPS enzymes, the protein could catalyse the addition of acylated PP groups to ACP. In all cases tested we observed smooth transfer of acylated PP in quantitative yield. The reaction allows the preparation of essential PKS and FAS intermediates. For example, malonyl ACP is required by all Type I and II synthases as the chain extending unit. Acetoacetyl ACP is an intermediate during both polyketide and fatty acid biosynthesis. The synthesis of hexanoyl NSAS *holo*-ACP (Fig. 4; NSAS = norsolorinic acid synthase) is important because this species is postulated to be the starter unit for norsolorinic acid **8** biosynthesis which is the precursor to the mycotoxin aflatoxin B_1 **9**.[24]



9 Aflatoxin B_1

The synthesis of acyl ACPs is important for the future study of the interaction of ACP-bound intermediates with other PKS components. Malonyl ACP can be generated by the acylation of purified *holo*-ACP either by self-acylation with malonyl CoA[25] or other β-keto-*N*-acetylcysteamyl thiol esters (SNACS) in the case of PKS ACPs,[26] or through catalysis with malonyl transfer enzymes for FAS *holo*-ACPs.[27] Some other acylated

Table 1 Observed (and calculated) molecular weights of *apo*-, *holo*- and acyl-ACP components. All errors are within ± 3 Da

ACP	*apo*	*holo*	Acetyl	Malonyl	Methyl-malonyl	Propionyl	*n*-Butyryl	Isobutyryl	Aceto-acetyl	Hexanoyl	Benzoyl
S. coel. act C17S	9100 (9101)	9441 (9441)	9484 (9483)	9527 (9525)	9544 (9541)	9497 (9497)	9510 (9511)	9512 (9511)	—	9541 (9539)	9548 (9545)
S. coel. FAS	8786 (8786)	9126 (9126)	9168 (9168)	9211 (9212)	9225 (9226)	9182 (9182)	9195 (9196)	9196 (9196)	9210 (9210)	9223 (9224)	9229 (9230)
E. coli FAS	8505 (8508)	8846 (8848)	—	—	—	—	—	—	—	—	—
S. rimosus Otc	9916 (9916)	10257 (10256)	—	—	—	—	—	—	—	—	—
Rat FAS	9813 (9817)	10157 (10158)	10198 (10199)	—	—	—	—	—	—	—	—
A. para. NSAS	14210 (14208)	14548 (14550)	—	14635 (14637)	—	—	—	—	—	14645 (14648)	—

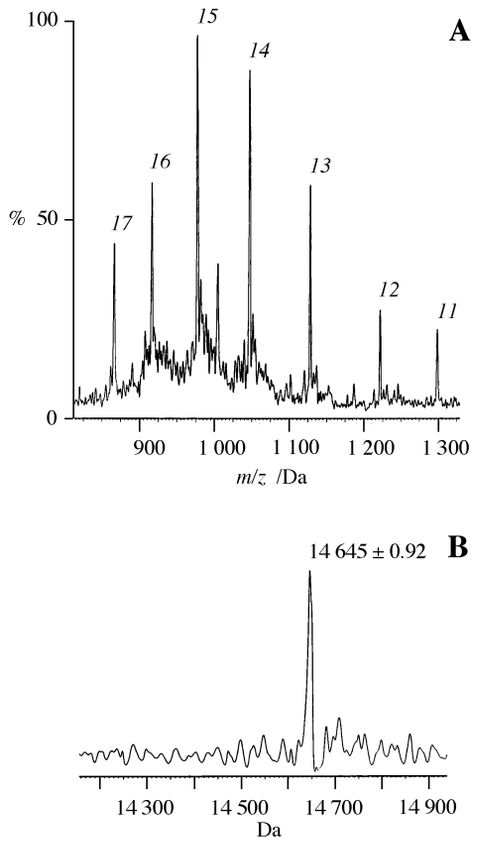


Fig. 4 ESMS analysis of *A. parasiticus* NSAS ACP domain after treatment with ACPS and hexanoyl CoA. **A**: raw MS data showing mass charge states. **B**: transform data. Calculated mass: hexanoyl NSAS-ACP 14 648 Da.

ACPs can be synthesised by reaction of *holo*-ACP with synthetic acyl imidazolides.[28] However, other acyl groups, such as acetoacetyl, cannot be transferred by either of these methods to FAS ACPs. *S. coelicolor* ACPS thus shows good potential for the preparation of a range of unusual ACP species which are difficult or impossible to access by other methods.

Two classes of PPTases have been recognised; broadly speaking these can be classified as those modifying FAS or PKS CPs (also known as ACPS) and those modifying NRPS CPs (Fig. 1). The archetypal FAS ACPS from *E. coli* is an enzyme which has been shown to be fairly intolerant in its substrate specificity for both ACP and CoA substrates.[14] Others in this class, such as the *S. pneumoniae* ACPS show similar limited properties.[29] On the other hand, *B. subtilis* Sfp (surfactin) PPTase from an NRPS has been shown to be rather more tolerant of substrate variation and this pattern is repeated for other similar enzymes such as the Svp PPTase from *Streptomyces verticillus*.[30] *S. coelicolor* ACPS most closely matches the *E. coli* ACPS family in terms of sequence. However in terms of its catalytic activity it appears that this enzyme more closely resembles the wide substrate specificity usually shown by the NRPS PPTases.

The *S. coelicolor* ACPS is thus of interest because of its unusually broad substrate specificity. For this reason *S. coelicolor* ACPS is a very useful catalyst for the production of acylated ACPs required for mechanistic exploration of polyketide biosynthesis. Furthermore these results show why *S. coelicolor* has been such a good host for heterologous expression studies of diverse polyketide synthases as it appears that the endogenous ACPS can activate a very wide range of synthase carrier protein components.

Experimental

Cloning of S. coelicolor ACPS. Two oligonucleotides were synthesised (University of Bristol Facility) complementary to the putative ACPS sequence: 5′-CTC GGA TCC CTA TCC CTC CGC GAT CAC CAC-3′ and 5′-CTC GGA TCC *CAT ATG* AGC ATC ATC GGG GTC GGG-3′ (*BamH1* sites underlined, *NdeI* italicised). Genomic DNA was prepared from *S. coelicolor* A3(2) by boiling and precipitation of denatured protein. The genomic DNA template was used in standard PCR reactions with the synthetic primers and *Taq* DNA polymerase. The reactions were repeated at Mg^{2+} concentrations of 1 mM and 2.5 mM. 35 Rounds of denaturation (96 °C, 60 s), annealing (55 °C, 60 s) and extension (72 °C, 30 s) were performed and the *ca*. 400 bp product was then ligated into predigested pGEM-T vector. The cloned insert was sequenced (LARK) and one clone lacking mutations was digested with *NdeI* and *BamH1*. The excised fragment was ligated between the *NdeI* and *BamH1* sites of pET15b (Novagen). This clone was designated pRJC012.

Expression and purification of *S. coelicolor* ACPS

pRJC012 was transformed into *E. coli* BL21(DE3) which was maintained on sterile L-agar containing carbenicillin (100 μg mL^{-1}). A single colony was transferred to sterile L-media (3 mL) containing carbenicillin (100 μg mL^{-1}) which was incubated at 37 °C, 200 rpm, for 2 h. The suspension was transferred to L-media (100 mL) containing carbenicillin (100 μg mL^{-1}) which was incubated at 37 °C 200 rpm until A_{590} reached 0.6–1.0. The culture broth was then stored at 4 °C overnight before centrifugation (4 500*g*, 10 min, 4 °C). The supernatant was discarded and fresh L-media added (100 mL). The suspension was aliquoted (10 × 5 mL) into fresh L-media (10 × 100 mL) containing carbenicillin (100 μg mL^{-1}) and incubation was continued at 37 °C, 200 rpm, until A_{590} reached 0.8–1.0. IPTG (isopropyl β-D-1-thiogalactopyranoside) was added to 1 mM and incubation was continued for 4 h. Cells were precipitated by centrifugation (4 500*g*, 10 min, 4 °C) and the supernatant was discarded. Aqueous buffer (50 mM Tris, pH 8.0, 100 mL) was added and the cell suspension was lysed by sonication at 0 °C. Cell solids were precipitated by centrifugation (11 200*g*, 15 min, 4 °C). The supernatant was filtered (0.4 μm) and then applied to a column of his-bind resin (Novagen) using a peristaltic pump at no more than 1 mL min^{-1}. The column was then eluted with binding buffer, then wash buffer according to manufacturers instructions. The column was then eluted with buffer containing imidazole and ACPS containing fractions (SDS-PAGE) were collected and combined. The combined fractions were then dialysed *vs*. EDTA buffer (50 mM EDTA, 50 mM Tris pH 8.5, twice) and then assay buffer (50 mM Tris, pH 8.8, twice). This procedure yielded 15–20 mg L^{-1} of ACPS. Gel filtration chromatography was performed using a Pharmacia Superdex-75 column (30 cm × 12 mm) eluted at 0.5 mL min^{-1} with Tris buffer pH 8.0 containing NaCl (150 mM). The column was calibrated with a commercial mixture of standard proteins (Sigma) before use. 0.1 mg of *S. coelicolor* his_6-ACPS was used for the analysis.

Assay conditions

ACP substrates were obtained by published methods.[18,22] Rat FAS ACP[23] and *A. parasiticus* norsolorinic acid synthase[24] ACP were obtained by standard cloning, expression and purification methods. All ACP substrates were purified and their purity checked by ESMS (Table 1) before use. Assay buffer consisted of 50 mM Tris, pH 8.8 containing Mg^{2+} (10 mM). Assays were performed at 30 °C for 120 min and contained ACPS (1 μM), ACP (40–80 μM) and CoA (or derivative, 1000 μM) in Assay buffer (300 μL).

Analysis

The ACP products were extracted by the addition of C_4-silica (Phenomenex Jupiter 15μ 300 Å, 3–5 mg).[31] The silica was

suspended in methanol (100 μL) before addition of the assay solution (100 μL). The mixture was vortexed for 2 min. The butylated silica particles were precipitated by centrifugation and the supernatant discarded. The pellets were washed with water (2 × 300 μL, 0.1% TFA) and then the ACP was eluted with CH_3CN (1 × 100 μL). The ACP solution was examined by ESMS as previously described.[18]

Acknowledgements

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