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Crystallization and preliminary X-ray analysis of the bacillaene synthase *trans*-acting acyltransferase PksC

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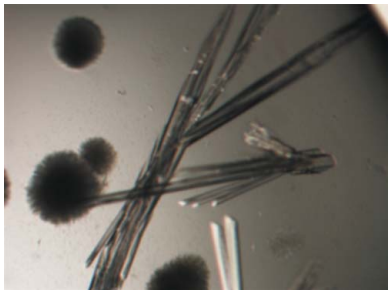

The antibiotic bacillaene is biosynthesized in *Bacillus subtilis* by a hybrid type 1 modular polyketide synthase/nonribosomal peptide synthetase of the *trans*-acyltransferase (*trans*-AT) class. Within this system, the essential acyl-group loading activity is provided by the action of three free-standing *trans*-acting acyltransferases. Here, the recombinant expression, purification and crystallization of the bacillaene synthase *trans*-acting acyltransferase PksC are reported. A diffraction data set has been collected from a single PksC crystal to 1.44 Å resolution and the crystal was found to belong to the orthorhombic space group $P2_12_12_1$.

1. Introduction

Polyketides (PKs), nonribosomal peptides (NRPs) and hybrids thereof (PK/NRPSs) constitute a diverse family of biologically important secondary metabolites that have found widespread application in human and veterinary medicine, agriculture and biotechnology (Walsh, 2008). Despite the astonishing structural and functional variety of these molecules, each is assembled in an analogous fashion involving the sequential addition of either organic acids or amino acids to an initial thiotemplate (Hertweck, 2009). For many products, biosynthesis is directed by the action of giant multifunctional enzymes (up to ∼5 MDa) of the type 1 modular polyketide synthase (PKS) class, in which the catalytic apparatus responsible for product assembly comprises a series of linearly arranged multidomain extension modules, with each module responsible for the elongation of the product chain by a single acyl or amino-acid unit (Fischbach & Walsh, 2006; Khosla *et al.*, 2009).

Historically, much of our understanding of modular synthases has derived from studies of secondary metabolic pathways from filamentous actinomyces and in particular the erythromycin-synthesizing deoxyerythromycin (DEBS) system (Katz, 2009). Recently, however, work focused on the characterization of secondary metabolic pathways from less well studied bacterial taxa has resulted in the identification of a novel group of multi-modular synthases which do not adhere to the established collinearity rules of the DEBS paradigm (Piel, 2010, and references therein). These novel PKSs, which are termed *trans*-AT synthases, seem to have evolved independently from other modular systems and comprise disparate enzymatic activities of apparently unrelated metabolic origin which appear to have converged to yield a single functional synthase. Unlike the DEBS-type canonical *cis*-AT systems, in which synthase evolution is guided by the duplication and diversification of extension modules and domains therein (Jenke-Kodama *et al.*, 2008), *trans*-AT systems appear to have arisen as a result of extensive horizontal gene transfer between bacteria, which accounts for their highly unusual biosynthetic frameworks, with aberrant module architectures, comprising novel catalytic domains and domain orders (Nguyen *et al.*, 2008). As is being increasingly realised, the potent biological activities of many *trans*-AT PKS products makes these molecules attractive targets for clinical development (Thomas *et al.*, 2010; Irschik *et al.*, 2007; Hale *et al.*, 2002).

In contrast to the canonical *cis*-AT modular systems, *trans*-AT synthases are characterized by the absence of individual acyltransferase domains within each PKS extension module, with this essential activity being provided through the action of between one and three free-standing *trans*-acting acyltransferases encoded within the synthase gene cluster. *Trans*-ATs act either as standalone functional domains, as fused tandem AT di-domains or as *trans*-acting enoyl reductase fusions (Cheng *et al.*, 2009). Detailed structural and functional characterization of *trans*-acting acyltransferases from *trans*-AT PKSs will not only provide significant insight into this novel biosynthetic activity, but may also reveal routes towards the exploitation and/or manipulation of *trans*-acting ATs for use in the generation of derivative polyketide products with improved pharmacological activities. Towards this goal, we have cloned, expressed and purified the *trans*-AT PksC from the prototypical *trans*-AT PKS bacillaene synthase. PksC has been subjected to crystallization screening and conditions supporting the growth of PksC crystals have been identified. A single diffraction data set to 1.44 Å resolution has been collected at Diamond Light Source.

2. Materials and methods

2.1. Gene cloning

The DNA sequence encoding PksC, a 288-amino-acid protein, was amplified from *Bacillus subtilis* 168 genomic DNA using the primers 5′-**CATATG**ATCACATATGTTTTTCCA-3′ (forward) and 5′-**CTCGAG**TTAGCGGGGCATTGCTTC-3′ (reverse), introducing appropriate restriction sites for subsequent cloning (*Nde*I and *Xho*I sites; bold). The PCR product was introduced into the pGEMT transfer vector (Promega), from which an *Nde*I/*Xho*I fragment was excised and ligated into pre-cut pET28a (Novagen; cut with the same enzymes). The sequence of the resulting construct (*pksC*::pET28a), encoding an N-terminally hexahistadine-tagged variant of PksC, was verified by DNA sequencing and transformed into *Escherichia coli* BL21 (DE3) cells for protein expression.

2.2. Protein expression, purification and characterization

100 ml Luria–Bertani (LB) medium supplemented with 50 µg ml^{-1} kanamycin was inoculated with a single colony of *pksC*::pET28a-transformed *E. coli* BL21 (DE3) cells and incubated overnight with shaking at 310 K. 20 ml of this culture was used to inoculate 1 l LB medium supplemented with kanamycin as above. The culture was grown until the optical density at 600 nm reached 0.6, at which point protein expression was induced by the addition of isopropyl β-D-1-thiogalactopyranoside to a final concentration of 1 m*M*. Following induction, the culture was grown for an additional 3–4 h before harvesting by centrifugation. The cell pellet was resuspended in His-load buffer (50 m*M* Na HEPES, 150 m*M* NaCl, 10 m*M* imidazole pH 7.5) supplemented with 5 m*M* 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride and lysed by ultrasonication. The resulting cell lysate was clarified by centrifugation and the supernatant liquid was applied onto a pre-equilibrated (in His-load buffer) 5 ml Hi-Trap chelating column (charged with nickel; GE Healthcare). PksC was eluted with an imidazole gradient (10–500 m*M*) over ten column volumes. Fractions containing protein (as identified by SDS–PAGE and $A_{280\,nm}$) were concentrated and injected onto a Hi-Load 16/60 Superdex 75 column (GE Healthcare) pre-equilibrated in 20 m*M* Tris–HCl, 150 m*M* NaCl pH 7.5. Eluted fractions containing PksC (as identified by SDS–PAGE and and $A_{280\,nm}$) were pooled and concentrated to 10 mg ml^{-1} by ultrafiltration. The purity of the final preparation was estimated to be >95% by SDS–PAGE analysis, with a yield of approximately 5 mg protein per gram of cell paste.

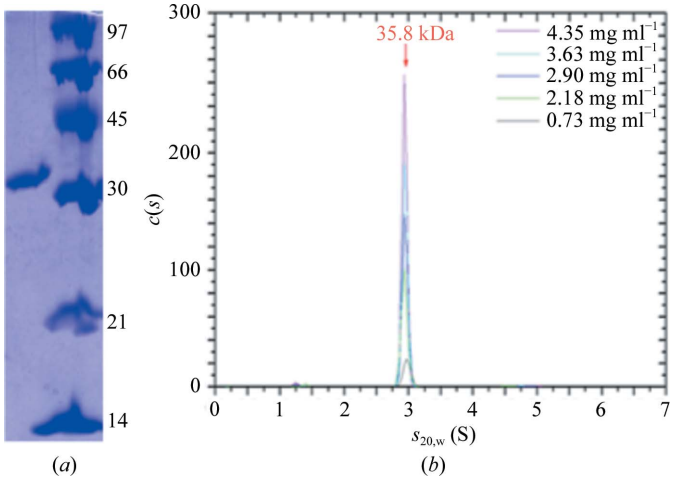

Figure 1
(*a*) 15% SDS–PAGE analysis of recombinant purified PksC. Molecular-weight markers are annotated in kDa. (*b*) Size-distribution [*c*(*s*)] analysis of PksC sedimentation-velocity data converted to standard conditions.

2.3. Analytical ultracentrifugation

Sedimentation-velocity (SV) experiments were carried out in a Beckman Coulter (Palo Alto, California, USA) ProteomeLab XL-I analytical ultracentrifuge (AUC) using both absorbance (at 280 nm) and interference optics. All AUC runs were performed at a rotation speed of 48 000 rev min^{-1} and an experimental temperature of 277 K. The sample volume was 400 µl for the SV experiments and the sample concentrations ranged between 1 and 5 mg ml^{-1} (nine samples were examined in this concentration range). The partial specific volume ($\bar{\nu}$) of the protein was calculated from its amino-acid sequence using the program *SEDNTERP* (Laue *et al.*, 1992) and was extrapolated to the experimental temperature using the method described by Durchschlag (1986). The density and viscosity of the buffer (20 m*M* Tris–HCl, 150 m*M* NaCl pH 7.5) at the experimental temperature were also calculated using *SEDNTERP*.

2.4. Crystallization and data collection

Initial crystallization screening was performed with commercially available screens from Qiagen using the sitting-drop vapour-diffusion technique. Crystallization experiments were set up using a Mosquito

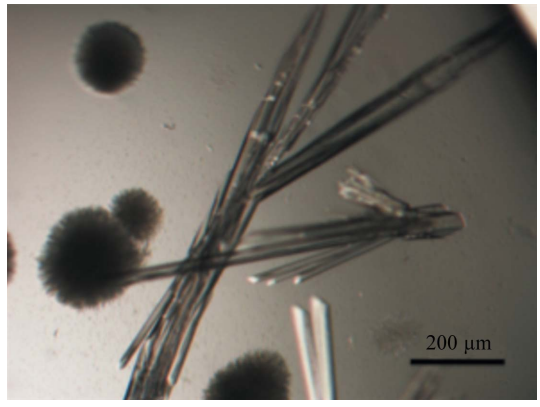

Figure 2
Photomicrograph of PksC crystals.

Table 1
Data-collection and processing statistics for PksC.

Values in parentheses are for the highest resolution shell.

Data collection	
X-ray source	I04, Diamond Light Source
Wavelength (Å)	0.9699
Temperature (K)	100
Detector	ADSC Quantum 315r CCD
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 53.19, b = 70.43, c = 76.28
Resolution range (Å)	37.09–1.44 (1.51–1.44)
V_M (Å^3 Da^{-1})	2.07
Solvent content (%)	40.6
Monomers in asymmetric unit	1
Data processing	
No. of observed reflections	354490
No. of unique reflections	53023
Completeness (%)	99.9 (99.9)
Mosaicity (°)	0.32
Multiplicity	6.7 (6.7)
R_{merge}† (%)	7.1 (30.6)
Mean $I/\sigma(I)$	16.6 (5.5)

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the reflection.

(TTP Labtech) nanolitre pipetting robot and MRC crystallization plates containing 100 µl each of the 96 conditions from the PACT screen (Newman *et al.*, 2005). 250 nl protein solution was pipetted into the sample well of each of the 96 crystallization-plate wells followed by 250 nl reservoir solution. Plates were sealed with clear sealing film and allowed to equilibrate at 294 K. Small PksC needle crystals were observed in a single condition, PACT 74 (20% PEG 3350, 0.2 *M* sodium bromide, 0.1 *M* Bis-Tris propane pH 7.5), after ∼2 d. Subsequent optimization using the hanging-drop vapour-diffusion method at 294 K, mixing 1 µl protein solution with 1 µl reservoir solution, resulted in the growth of large PksC crystal clusters comprising multiple rod-shaped crystals in crystallization solutions comprising 17–22% PEG 3350, 0.2 *M* sodium bromide, 0.1 *M* bis-tris propane pH 9.5 that achieved maximum dimensions (50 × 600 µm) in around one week. Single rod-shaped PksC crystals were separated from the clusters using an acupuncture needle, cryoprotected in well solution supplemented with 25% glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at Diamond Light Source, England (station I04 equipped with an ADSC Quantum 315r CCD detector). Crystals were maintained at cryogenic temperature during data collection. All data were processed with *MOSFLM* (Leslie, 2006) and scaled using *SCALA* (Evans, 2006) as implemented in the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994).

3. Results and discussion

An N-terminally hexahistadine-tagged version of the bacillaene synthase *trans*-acyltransferase PksC has been successfully cloned and recombinantly overexpressed in *E. coli*. Soluble recombinant PksC has been purified to homogeneity using a combination of nickel-affinity His-chelation and size-exclusion chromatography (Fig. 1). Initial characterization by analytical ultracentrifugation revealed this material to be a monodisperse single species with a native molecular mass of 35.8 kDa (Fig. 1), implying that PksC is monomeric in solution. Diffraction-quality crystals were grown using the hanging-drop vapour-diffusion method with a well solution consisting of 17–22% PEG 3350, 0.2 *M* sodium bromide, 0.1 *M* Bis-Tris propane pH 9.5 (Fig. 2). A single X-ray diffraction data set was collected on beamline I04 at Diamond Light Source, with the crystal yielding good diffraction images. Crystal parameters and diffraction data statistics are summarized in Table 1. The crystal belonged to the orthorhombic space group $P2_12_12_1$, as determined using the autoindexing routine of *MOSFLM* (Leslie, 2006) and *SCALA* (Evans, 2006) from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) and inspection of systematic absences, with unit-cell parameters as given in Table 1. The asymmetric unit contained one molecule of PksC, giving a crystal volume per protein mass (V_M) of 2.07 Å^3 Da^{-1} and a solvent content of 40.6% (Matthews, 1968). A complete structure determination of PksC is currently under way in order to provide insight into the catalytic activity and substrate specificity of this enzyme.

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