Crystallization and preliminary X-ray crystallographic analysis of a putative nonribosomal peptide synthase AmbB from *Pseudomonas aeruginosa*

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AmbB is a putative nonribosomal peptide synthase from *Pseudomonas aeruginosa*, which is involved in the production of IQS, a potent cell–cell communication signal molecule that integrates the quorum-sensing mechanism and stress response. It consists of 1249 amino acids and contains an AMP-binding domain, a phosphopantetheine-binding (PB) domain and a condensation (C) domain. In this report, a truncated form of AmbB that contains the PB domain and the condensation domain was overexpressed with an N-terminal GST tag in *Escherichia coli* and purified as a monomer using affinity and size-exclusion chromatography. The recombinant AmbBc (comprising residues 727–1249 of full-length AmbB) was crystallized using the hanging-drop vapour-diffusion method and a full data set was collected to 2.45 Å resolution using a synchrotron-radiation source. The crystals belonged to space group *P*6₁22 or *P*6₅22, with unit-cell parameters $a = b = 87.81$, $c = 286.8$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$, and contained one molecule per asymmetric unit.

1. Introduction

Bacteria are highly social organisms and are capable of cell-to-cell communication *via* quorum sensing (QS), which mainly coordinates various bacterial community activities including virulence and biofilm formation (Fuqua & Greenberg, 2002; Federle & Bassler, 2003). *Pseudomonas aeruginosa*, which is responsible for chronic pneumonia in cystic fibrosis patients, has evolved a complicated regulatory QS network to regulate bacterial virulence. Three QS systems (*las*, *rhl* and *pqs*) that function in a hierarchical manner have been identified in *P. aeruginosa* (Venturi, 2006; Williams & Cámara, 2009). Among them, the *las* system controls the expression of the *rhl* and *pqs* genes, which is at the top of the regulatory networks (Pesci *et al.*, 1999; de Kievit *et al.*, 2002; Gilbert *et al.*, 2009).

However, the central role of the *las* system in controlling the *rhl* and *pqs* QS systems is dependent on growth and environmental conditions. For example, phosphate-depletion stress has recently been found to favour *las*-independent activation of the *rhl* and *pqs* QS systems (Jensen *et al.*, 2006; Dekimpe & Déziel, 2009; Smith *et al.*, 2006). More importantly, clinical isolates frequently contain loss-of-function mutations in the central *las* system (Smith *et al.*, 2006; Tingpej *et al.*, 2007; Wilder *et al.*, 2009; Karatuna & Yagci, 2010; Hoffman *et al.*, 2009). Investigation of the mechanism that may functionally substitute for *las* led to the identification of a new QS signal, 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS), that integrates QS and stress response (Lee *et al.*, 2013).

L-2-Amino-4-methoxy-*trans*-3-butenic acid (AMB; methoxy-vinylglycine) is a secondary metabolite which is thought to be a potent antibiotic (Sahm *et al.*, 1973; Scannel *et al.*, 1972; Tisdale, 1980; Berkowitz *et al.*, 2006). A five-gene cluster *ambABCDE* has been identified as being involved in AMB biosynthesis and exportation using biochemical assays combined with site-directed mutagenesis in strain PAO of *P. aeruginosa* (Lee *et al.*, 2010). However, a recent study revealed that the *ambBCDE* gene cluster is involved in the production of IQS rather than AMB biosynthesis (Lee *et al.*, 2013), as deletion of either *ambB*, *ambD*, *ambC* or *ambE* led to reduced production of IQS, while a null mutation of *ambA* had no effect on IQS production (Lee *et al.*, 2013). IQS modulates AMB production

through the *pqs* and *rhl* QS systems and is essential for full bacterial virulence (Lee *et al.*, 2013).

AmbC and AmbD are putative dioxygenases with over 42% identity to each other, while AmbB and AmbE are putative non-ribosomal peptide synthases (NRPS) that share ~24% sequence identity but have different domain architectures (Lee *et al.*, 2010). AmbB contains an AMP-binding domain, a phosphopantetheine-binding (PB) domain and a condensation (C) domain (Fig. 1). The condensation domain is responsible for amide-bond formation, which is the central chemical step in nonribosomal peptide synthesis including IQS synthesis (Stachelhaus *et al.*, 1998).

As a first step towards the structural elucidation of the molecular mechanism underlying IQS production, we overexpressed, purified and crystallized a truncated form of AmbB containing the PB domain and the C domain. A 2.45 Å resolution X-ray diffraction data set has been collected.

2. Materials and methods

2.1. Gene cloning and expression

The *ambB* gene (PA2305) encoding a truncated AmbB (UniProt entry Q9I1H0) containing the PB and C domains (AmbBc; amino acids 727–1249 containing a V733A mutation) was amplified from *P. aeruginosa* PAO1 genomic DNA by PCR using the forward primer 5'-CGGGATCCACCGCGCCGAGCC-3' and the reverse primer 5'-CCGCTCGAGTTAGGAAGCGTTGCAGCCC-3', and inserted into *Bam*HI and *Xho*I restriction-endonuclease sites in pGEX-6p-1 vector (GE Healthcare), with a GST tag at the N-terminus. AmbBc was expressed as a GST-fusion protein in *Escherichia coli* strain BL21 (DE3) CodonPlus. The cells were grown in LB medium at 310 K supplemented with ampicillin (0.1 mg ml⁻¹) and chloramphenicol (0.05 mg ml⁻¹). Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.1 mM when the optical density at

600 nm reached ~0.6 and the cells were incubated overnight at 291 K. The cells were harvested by centrifugation for 30 min at 3220g and stored at 193 K for protein purification.

2.2. Protein purification

The frozen cells were thawed at room temperature and resuspended in lysis buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM DTT) containing 2 mM benzamidine, 100 μM PMSF, 1 mg ml⁻¹ hen egg-white lysozyme. The resuspended cells were incubated on ice for 30 min and then broken using a Soniprep (Sanyo). The lysed cells were centrifuged at 40 555g at 277 K for 30 min.

The clarified cell lysate was mixed with Glutathione Sepharose beads (GE Healthcare) pre-equilibrated with lysis buffer for 1 h. The unbound proteins were then washed away with lysis buffer after loading onto the column. The GST-AmbBc was eluted with lysis buffer containing 20 mM reduced glutathione. The eluted GST-AmbBc was mixed with PreScission protease at a ratio of 50:1(w:w) and incubated at 277 K overnight to cleave off the GST tag. After desalting using a HiPrep Desalting column (GE Healthcare) with desalting buffer (20 mM Tris-HCl pH 8.0, 500 mM NaCl, 2 mM DTT), the protein sample was passed through a Glutathione Sepharose column to remove the cleaved GST fragment and other contaminants. The flowthrough fractions were concentrated and applied onto a Superdex 200 column (HiLoad 26/60 Prep Grade, GE Healthcare) pre-equilibrated with a buffer consisting of 20 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM DTT. The fractions containing AmbBc were pooled and concentrated to a final concentration of 11 mg ml⁻¹ and stored at 193 K.

2.3. Crystallization

Initial crystallization trials were carried out by the hanging-drop vapour-diffusion method in 96-well crystallization plates at both 277

Figure 1
Conserved domains of AmbB predicted by the SMART program (Letunic *et al.*, 2011).

Figure 2
(a) Clusters of needle-like crystals after screening. (b) The rod-like crystals obtained using the detergent MEGA-9. (c) Optimized AmbBc crystals used for data collection.

Table 1

Data-collection and processing statistics for AmbBc crystals.

Values in parentheses are for the outermost resolution shell.

Beamline, synchrotron	BL17U, SSRF, Shanghai, China
Wavelength (Å)	0.9793
Detector	ADSC Q315
Data-collection temperature (K)	100
Crystal-to-detector distance (mm)	320
Rotation range per image (°)	0.5
Total rotation range (°)	100
Exposure time per image (s)	1
Resolution range (Å)	59.0–2.45
Space group	$P6_122$ or $P6_522$
Unit-cell parameters (Å, °)	$a = 87.81, b = 87.81, c = 286.79,$ $\alpha = 90, \beta = 90, \gamma = 120$
Total No. of measured intensities	273037
Unique reflections	24816
Mosaicity	0.50
Completeness (%)	99.6 (99.8)
Multiplicity	11.0 (11.5)
$R_{\text{merge}}^{\dagger}$ (%)	11.6 (93.0)
$R_{\text{meas}}^{\ddagger}$ (%)	12.7 (96.0)
$\langle I/\sigma(I) \rangle$	14.7 (3.2)
Matthews coefficient (Å ³ Da ^{−1})	2.74
Solvent content (%)	55.0

$\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of an individual reflection and $\langle I(hkl) \rangle$ is the average intensity of that reflection. $\ddagger R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $N(hkl)$ is the multiplicity, $I_i(hkl)$ is the intensity of the i th measurement of reflection hkl and $\langle I(hkl) \rangle$ is the average value over multiple measurements.

and 289 K. A total of 960 conditions were screened using commercially available crystallization screening kits (Qiagen) and the Phoenix crystallization robot (Art Robbins Instruments). A typical drop consisting of 200 nl protein solution mixed with 200 nl precipitant solution was equilibrated against 60 µl reservoir solution. Initial hits were obtained after 7 d in three conditions [0.1 M ammonium sulfate, 0.1 M HEPES pH 7.5, 10% PEG 4000 from The MbClass II Suite at 277 K; 0.1 M HEPES pH 7.5, 10% PEG 8000, 10% ethylene glycol (EG) of The PEGs II Suite at 277 K; and 0.85 M HEPES pH 7.5, 8.5% PEG 8000, 15% glycerol from The Cryos Suite at 289 K].

Subsequent optimizations were performed manually by mixing 2 µl protein solution with 2 µl precipitant solution and equilibrating against 500 µl reservoir solution using the hanging-drop vapour-diffusion technique in 24-well crystallization plates at 277 K. Several methods including additive screening and microseeding were used for optimization but without obvious effects on crystal morphology (Fig. 2a). Finally, MEGA-9 from the detergent screening kit (Hampton Research) was found to change the crystal form (Fig. 2b). The rod-shaped crystals (30 × 40 × 300 µm) used for data collection (Fig. 2c) were obtained from a condition consisting of 0.1 M Tris pH 7.5, 11% PEG 6000, 2.5% EG/MPD, 25 mM MEGA-9.

2.4. Data collection and processing

Crystals were harvested with cryoloops (CrystalCap HT) and transferred from the mother liquor into cryoprotectant solution consisting of the reservoir solution supplemented with 20% (v/v) glycerol. All crystals were flash-cooled in liquid nitrogen and exposed to X-rays at 100 K. X-ray data sets were collected on beamline BL17U at the Shanghai Synchrotron Radiation Facility (SSRF) in China. A total of 400 diffraction images with 0.5° oscillation per image and an exposure time of 1 s per image were collected. The crystal diffracted to better than 2.5 Å resolution (Fig. 3). Diffraction images were indexed, integrated and scaled using *HKL-2000* (Otwinowski & Minor, 1997). Data-collection and processing statistics are summarized in Table 1.

3. Results and discussion

We have expressed and purified AmbBc using GST-affinity and size-exclusion chromatography (SEC). The SEC elution profile showed a single peak (Fig. 4a) at an apparent molecular weight (MW) of 57 kDa, which is close to the calculated MW of 55 799.70 Da, suggesting that AmbBc may exist as a monomer in solution. SDS-PAGE with Coomassie staining showed that purified AmbB is at least

Figure 3

A representative X-ray diffraction pattern of an AmbBc crystal. The outer circle indicates a resolution of 2.5 Å.

Figure 4
Purification of AmbBc. (a) Superdex-200 gel-filtration column chromatogram showing a peak at an apparent molecular mass of 57 kDa; (b) SDS-PAGE gel of purified AmbBc showing a final purity of ~95%. The left lane contains molecular-mass marker (labelled in kDa).

95% pure (Fig. 4b). About 8 mg pure AmbBc could be obtained from 2 l cell culture. The identity of the purified AmbBc has been confirmed by mass spectrometric analysis.

Initially, clusters of conjoined thin needle-like crystals of AmbBc were observed. However, all attempts to obtain larger and single crystals failed until we used the detergent screening kit to identify MEGA-9, which changed the crystal morphology from a needle-like to a rod-like shape (Fig. 2b). A complete data set to 2.45 Å resolution (Fig. 3, Table 1) was collected from a single crystal using a synchrotron-radiation source. The crystals belonged to space group $P6_22$ or $P6_522$, with unit-cell parameters $a = b = 87.81$, $c = 286.79$ Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$. There is one molecule per asymmetric unit, giving a solvent content of 55.0% and a Matthews coefficient of $2.74 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968).

As AmbB has no structural homologue available in the Protein Data Bank, heavy-atom soaks were performed with Heavy Atom

Screen M1 (Hampton Research). Crystals were soaked in heavy-atom solutions at concentrations of 0.1–0.5 mM overnight and of 1 mM for 2 and 4 h. In addition, we expressed and purified selenomethionine-substituted AmbBc and set up crystallization using the same condition as that used for the native protein. Further crystallization and structural determination of AmbBc using the single-wavelength anomalous dispersion method is under way.

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