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Expression, crystallization and preliminary crystallographic analysis of $PilZ_{XAC1133}$ from *Xanthomonas axonopodis* pv. *citri*

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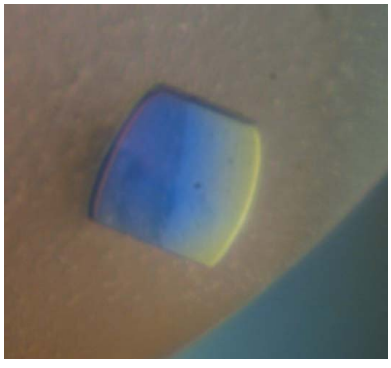
Proteins containing PilZ domains are widespread in Gram-negative bacteria and have recently been shown to be involved in the control of biofilm formation, adherence, aggregation, virulence-factor production and motility. Furthermore, some PilZ domains have recently been shown to bind the second messenger bis(3′→5′)cyclic diGMP. Here, the cloning, expression, purification and crystallization of $PilZ_{XAC1133}$, a protein consisting of a single PilZ domain from *Xanthomonas axonopodis* pv. citri, is reported. The closest $PilZ_{XAC1133}$ homologues in *Pseudomonas aeruginosa* and *Neisseria meningitidis* control type IV pilus function. Recombinant $PilZ_{XAC1133}$ containing selenomethionine was crystallized in space group $P6_1$. The unit-cell parameters were a = 62.125, b = 62.125, c = 83.543 Å. These crystals diffracted to 1.85 Å resolution and a MAD data set was collected at a synchrotron source. The calculated Matthews coefficient suggested the presence of two $PilZ_{XAC1133}$ molecules in the asymmetric unit.

1. Introduction

Bacteria from the genus *Xanthomonas* infect many plant hosts, including crops of economic importance, and *Xanthomonas axonopodis* pv. *citri* (Xac) causes citrus canker in a wide variety of citrus species (Brunings & Gabriel, 2003). The sequencing of the Xac genome (Da Silva *et al.*, 2002) revealed the existence of a large number of proteins containing GGDEF, HD-GYP, EAL and PilZ domains (Andrade *et al.*, 2006). These domains have been implicated in the production (GGDEF), degradation (HD-GYP and EAL) and binding (PilZ) of the second messenger bis(3′→5′)cyclic diGMP (c-diGMP; Paul *et al.*, 2004; Simm *et al.*, 2004; Ryan *et al.*, 2006; Pratt *et al.*, 2007). C-diGMP signalling is now recognized as extremely important for a variety of complex bacterial phenomena including biofilm formation, virulence, motility, pilus expression and photosynthesis (Römling *et al.*, 2005). The only high-resolution structures of PilZ domains available are the NMR structure of the *Pseudomonas aeruginosa* protein Pa4608 (Ramelot *et al.*, 2007) and crystal structures of the PilZ domain-containing protein VCA0042 from *Vibrio cholerae* in the presence and absence of c-diGMP (Benach *et al.*, 2007).

PilZ domains typically contain around 120 residues and make up a superfamily that is highly diverse at the level of primary structure and that can be divided into many paralogous groups (Amikam & Galperin, 2006; Benach *et al.*, 2007). The Xac genome codes for four proteins with PilZ domains, one of which is XAC1133, or $PilZ_{XAC1133}$, which is coded by the *pilZ* gene (Da Silva *et al.*, 2002). The closest $PilZ_{XAC1133}$ homologues in *P. aeruginosa* and *Neisseria meningitidis* control type IV pilus (T4P) function, although perhaps in different ways. In *P. aeruginosa*, PilZ is necessary for T4P biogenesis and TP4-dependent twitching motility (Alm *et al.*, 1996). On the other hand, a *pilZ* knockout in *N. meningitidis*, although piliated, is not able to produce T4P-dependent bacterial aggregates (Carbonnelle *et al.*, 2005).

In this report, we describe the cloning and expression of recombinant $PilZ_{XAC1133}$ containing selenomethionine and the collection of



a multiwavelength anomalous dispersion (MAD) data set that is being used to determine its three-dimensional structure.

2. Cloning and expression of PilZ$_{XAC1133}$

PilZ$_{XAC1133}$ was amplified by PCR from Xac genomic DNA (Da Silva *et al.*, 2002) using the primers 5′-TTCTCCATGGACCATATGAGT-GCAATGAAT-3′ and 5′-CGGGATCCCTCGAGTTACATCGTAT-GCGTCG-3′. The PCR product codes for the full-length protein (117 amino acids; 12.3 kDa). It was digested with *Nde*I and *Bam*HI and subcloned into the pET-3a expression vector (Studier *et al.*, 1990) previously digested with the same endonucleases. PilZ$_{XAC1133}$ was expressed in *Escherichia coli* strain BL21 (DE3) (Studier *et al.*, 1990). Selenomethionine-containing protein was produced by growing a 500 ml culture in M9 medium to an OD$_{600\,nm}$ of 0.8 at 310 K, at which point 100 mg l^{-1} lysine, 100 mg l^{-1} phenylalanine, 100 mg l^{-1} threonine, 50 mg l^{-1} isoleucine, 50 mg l^{-1} valine and 60 mg l^{-1} selenomethionine were added (Berne *et al.*, 1999). After 15 min, 0.4 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG) was added and the cells were grown for 4 h before sedimentation and storage at 193 K.

3. Protein purification

Cells from a 1 l culture were resuspended in 25 ml 50 m*M* Tris–HCl pH 8.0, 25% sucrose, 1 m*M* EDTA and lysed using a French press. The soluble fraction was applied onto an SP-Sepharose Fast Flow (FF) HiLoad 16/10 column (Amersham Pharmacia) previously equilibrated with 50 m*M* Tris–HCl pH 8.0 and 1 m*M* EDTA. Bound proteins were eluted using a 0–1 *M* NaCl gradient over 12 column volumes. The fractions containing the purest samples of PilZ$_{XAC1133}$ were pooled. In spite of the fact that the purification only involved one chromatographic step, the elevated pI value of the protein (theoretical value of 9.05) allowed us to obtain an adequate number of fractions that had very little contaminant visible in an overloaded Coomassie-stained SDS polyacrylamide gel. The purity, as estimated by visual inspection of the gel, was above 95%. The protein solution was then dialyzed against 5 m*M* Tris–HCl pH 7.0 and concentrated to 5–10 g l^{-1} using Centricon (Millipore) concentrators with a 3 kDa membrane cutoff.

4. Crystallization

Initial crystallization conditions were screened by the sparse-matrix sampling approach using Crystal Screen and Index Screen (Hampton Research) matrices. Initially, crystals were obtained under several conditions of vapour diffusion using the sitting-drop technique at 291 K. Optimization of the crystallization conditions was then achieved by modification of the concentration of the precipitating reagent, the buffer pH and the temperature. The best crystals were obtained by mixing equal volumes (1.5 µl) of a 7.2 mg ml^{-1} protein solution in 5 m*M* Tris–HCl pH 7.0 with reservoir solution consisting of 24%(*w*/*v*) PEG 4000, 0.1 *M* Tris–HCl pH 8.0 and 0.2 *M* $MgCl_2$. Crystals were initially grown at 281 K for one month, which was followed by transfer to 291 K to obtain mature-sized crystals (Fig. 1). The crystal was frozen immediately before data collection in a stream of nitrogen at 100 K. No cryoprotectant was used.

5. Data collection

Data were collected on the W01B-MX2 beamline of the Laboratório Nacional de Luz Síncrotron, Campinas, São Paulo using a MAR Mosaic 225 CCD detector. Crystals were flash-frozen and maintained at 100 K in a stream of cold nitrogen gas during measurement. MAD data sets were collected using a single crystal at the three wavelengths 0.97829, 0.97818 and 1.03448 Å, corresponding to peak, inflection and remote points of the fluorescence spectrum of the PilZXAC1133 crystal, respectively. The f' and f'' anomalous scattering factors shown in Table 1 were estimated from the fluorescence spectrum using the *CHOOCH* software (Evans & Pettifer, 2001). Diffraction intensities for the data sets were integrated and scaled using the programs *DENZO* and *SCALEPACK*, respectively (Otwinowski & Minor, 1997). The Friedel mates were scaled separately during data processing to calculate the anomalous signal.

Table 1
Crystal parameters and data-reduction statistics.

Data sets were measured from a single crystal. Values in parentheses are for the highest resolution shell. Each member of a Friedel pair was counted as a separate reflection.

	Peak	Peak	Inflection	Remote
Space group	$P6_1$	$P6_122$	$P6_122$	$P6_122$
Unit-cell parameters				
a (Å)	62.125	62.125	62.168	62.202
b (Å)	62.125	62.125	62.168	62.202
c (Å)	83.543	83.542	83.605	83.658
Resolution range (Å)	40.00–1.85 (1.92–1.85)	40.00–1.86 (1.93–1.86)	40.00–1.95 (2.02–1.95)	40.00–1.95 (2.02–1.95)
No. of observed reflections	328014	323942	211604	273081
No. of unique reflections	30576	15168	13219	13262
$\langle I/\sigma(I)\rangle$	24.7 (1.74)	34.01 (2.60)	33.63 (7.46)	25.6 (5.3)
Multiplicity	10.7 (5.9)	21.4 (11.9)	16.0 (15.9)	20.6 (20.4)
Completeness (%)	99.0 (92.1)	99.7 (97.8)	99.8 (100.0)	99.9 (100.0)
R_{merge}† (%)	8.6 (60.1)	8.7 (55.8)	8.6 (43.1)	11.6 (62.5)
No. of images	360	360	248	319
Oscillation angle (°)	1	1	1	1
Wavelength (Å)	0.97829	0.97829	0.97818	1.03448
f'/f''	6.43/−7.81	6.43/−7.81	3.56/−10.39	3.60/−2.80

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$.

The crystal diffracted to 1.85 Å resolution in space group $P6_1$. We note that the MAD data set was initially processed in space group $P6_122$ (Table 1) and these data were used to obtain initial estimates of phases and to initiate model building (to be published elsewhere). However, refinement convergence could not be achieved without reducing the space-group symmetry to $P6_1$ (to be published elsewhere). For this reason, Table 1 shows statistics for the processing of the MAD data set in $P6_122$ for all three wavelengths and the statistics for the peak data set after reprocessing in $P6_1$. There are two

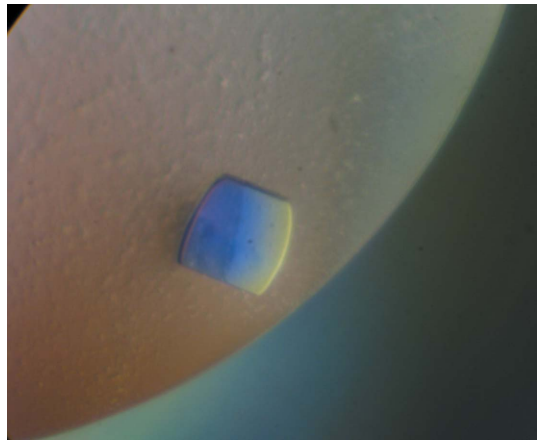


Figure 1
Crystal of PilZXAC1133, with approximate dimensions of 0.15 × 0.1 × 0.10 mm.

PilZ$_{XAC1133}$ monomers per $P6_1$ asymmetric unit (Matthews coefficient V_M = 1.9 Å^3 Da^{-1}) and the estimated solvent content is 33.8%.

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