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Cloning, purification, crystallization and preliminary crystallographic analysis of a penicillin-binding protein homologue from *Pyrococcus abyssi*

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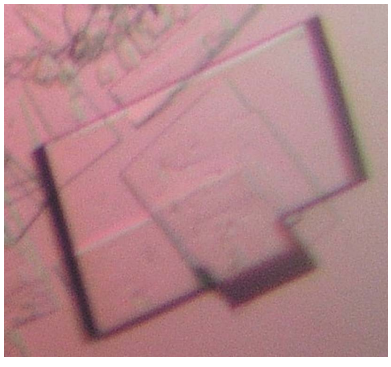


The genome of the hyperthermophilic archaeon *Pyrococcus abyssi* contains a gene (*pab0087*) encoding a penicillin-binding protein (PBP) homologue. This sequence consists of 447 residues and shows significant sequence similarity to low-molecular-weight PBPs and class C β-lactamases. The Pab0087 protein was overexpressed, purified and crystallized. Diffraction data from two different crystal forms were collected to 2.7 and 2.0 Å resolution. Both crystals belong to space group $C2$, with unit-cell parameters a = 160.59, b = 135.74, c = 113.02 Å, β = 117.36° and a = 166.97, b = 131.25, c = 189.39 Å, β = 113.81°, respectively. The asymmetric unit contains four and eight molecules, respectively, with fourfold non-crystallographic symmetry.

1. Introduction

In bacteria, the last step in cell-wall biosynthesis is catalyzed by D,D-transpeptidases. These enzymes are members of the family of penicillin-binding proteins (PBPs), wich are separated into two groups: high-molecular-weight PBPs and low-molecular-weight PBPs (Ghuysen, 1991). PBPs are the essential target of β-lactams, which are analogues of their natural substrate D-Ala:D-Ala (Tipper & Strominger, 1965). This class of antibiotics acts by inactivating PBPs through acylation of their catalytic serine.

Production of β-lactamases is the major resistance mechanism that has emerged as a result of intensive use of β-lactam antibiotics. These enzymes catalyze the irreversible hydrolysis of the β-lactam ring, thus yielding inactive antibiotics. Based on their primary sequence, β-lactamases are separated into four molecular classes: A, B, C and D (Ambler, 1980). Classes A, C and D are active serine enzymes and follow a catalytic pathway similar to that of PBPs. Class B β-lactamases are zinc metalloenzymes.

Low-molecular-weight PBPs and β-lactamases are both members of the penicillin-recognizing protein family (PRPs) and exhibit the same overall structural fold: they are composed of a single domain containing two regions, one α/β and one all-helical, with the catalytic site lying between them. PRPs also possess three conserved sequence motifs close to the active-site serine which are directly or indirectly involved in substrate recognition and in the catalytic pathway (S-*X*-*X*-K containing the active serine, Y/S-*X*-N and K/H-T-G).

Pyrococcus abyssi is a hyperthermophilic archaeon with an optimal growth temperature of 369 K (Erauso *et al.*, 1993) and its genome has been completely sequenced (Cohen *et al.*, 2003). Analysis of the genome revealed the presence of an amino-acid sequence homologous to those of low-molecular-weight PBPs and class C β-lactamases (Lecompte *et al.*, 2001). This protein of 50.4 kDa, called Pab0087, shows an amino-acid sequence identity of 20–25% with PBPs and class C β-lactamases and possesses the three conserved catalytic elements. Currently, little information is available about the archaeal cell wall (Kandler & König, 1998), but the homology of Pab0087 to PBPs suggest that it may play a role in the biosynthesis of a pseudopeptidoglycan in archaea. Thus, the three-dimensional structure of Pab0087 will provide for the first time data about a non-bacterial PRP member and will further our understanding of the evolution of the PRP family as well as of archaeal cell-wall biosynthesis.

Here, we describe the cloning, expression, purification and crystallization of the *P. abyssi* Pab0087 protein. Preliminary X-ray analyses are reported for two crystal forms, both belonging to space group *C*2.

2. Materials and methods

2.1. Cloning, expression and purification

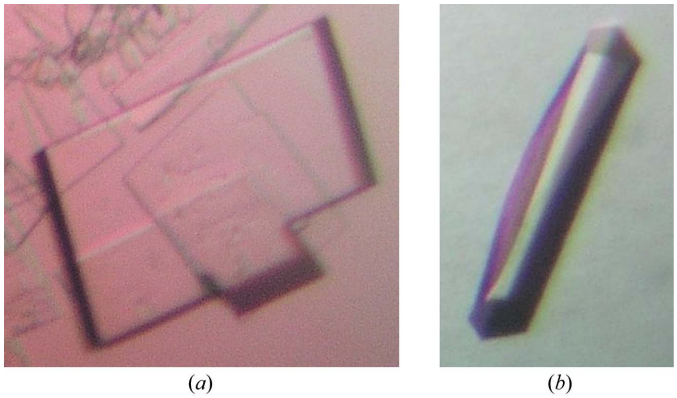
The predicted open reading frame encoding the hypothetical β-lactamase Pab0087 was amplified by PCR from genomic DNA of *P. abyssi*. The PCR-amplified fragment was cloned into the pPCR-Script Cam SK(+) cloning vector (Stratagene). The DNA was subcloned into pET29a (Novagen). *Escherichia coli* BL21-CodonPlus (DE3) RIL (Stratagene) cells freshly transformed with pET29a/*pab0087* were grown at 310 K overnight in LB medium containing 30 µg ml^{-1} kanamycin and 40 µg ml^{-1} chloramphenicol. At an OD_{600} of 0.6, IPTG was added to a final concentration of 1 m*M* and cells were grown at 310 K for 3 h. Following harvesting, the cell pellet was resuspended in 50 m*M* Tris–HCl buffer pH 8.5 and lysed by sonication. The cell lysate obtained by centrifugation at 27 000*g* for 20 min was heated at 363 K for 15 min and then centrifuged for 1 h at 27 000*g*. The supernatant was treated with spermine at a final concentration of 14 m*M*. After an additional centrifugation for 15 min at 12 000*g*, the supernatant was filtered (Minisart 0.45 µm, Sartorius) and concentrated (Amicon Ultra 10K 15 ml, Millipore). This extract was injected onto a 5 ml Q-Sepharose Hi-Trap column pre-equilibrated in 50 m*M* Tris–HCl buffer pH 8.5. The protein Pab0087 was eluted with a linear gradient of 0–1 *M* NaCl and eluted at 300 m*M*. Fractions containing Pab0087 were pooled, concentrated and then injected onto a Superdex 75 HR 10/30 gel-filtration column (Amersham Biosciences) pre-equilibrated in 50 m*M* Tris–HCl buffer pH 8.5, 300 m*M* NaCl. The protein Pab0087 was eluted with an apparent molecular weight of approximately 112 kDa, indicating that Pab0087 is a dimer under these conditions.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

	Pab0087 form 1	Pab0087 form 2
X-ray source	Micromax 7 rotating anode	FIP-BM30A
Space group	*C*2	*C*2
Unit-cell parameters (Å, °)	a = 160.59, b = 135.74, c = 113.02, β = 117.36	a = 166.99, b = 131.27, c = 189.41, β = 113.81
Wavelength (Å)	1.5418	0.97976
Resolution range (Å)	50–2.7 (2.8–2.7)	20–2.0 (2.12–2.0)
No. of observations	517326 (52727)	934274 (141608)
Unique reflections	58345 (5933)	244085 (38374)
Completeness (%)	98.8 (98.3)	97.0 (95.4)
Redundancy	8.86 (8.88)	3.83 (3.69)
$\langle I/\sigma(I)\rangle$	24.1 (6.8)	26.58 (5.93)
R_{sym}† (%)	7.9 (33.3)	3.9 (32.2)
Subunits per ASU	4	8
V_M (Å^3 Da^{-1})	2.71	2.37
Unit-cell volume (Å^3)	2.2 × 10^6	3.8 × 10^6

† $R_{sym} = \sum |I - \langle I\rangle| / \sum I$, where I is the intensity of an individual reflection and $\langle I\rangle$ is its mean value.

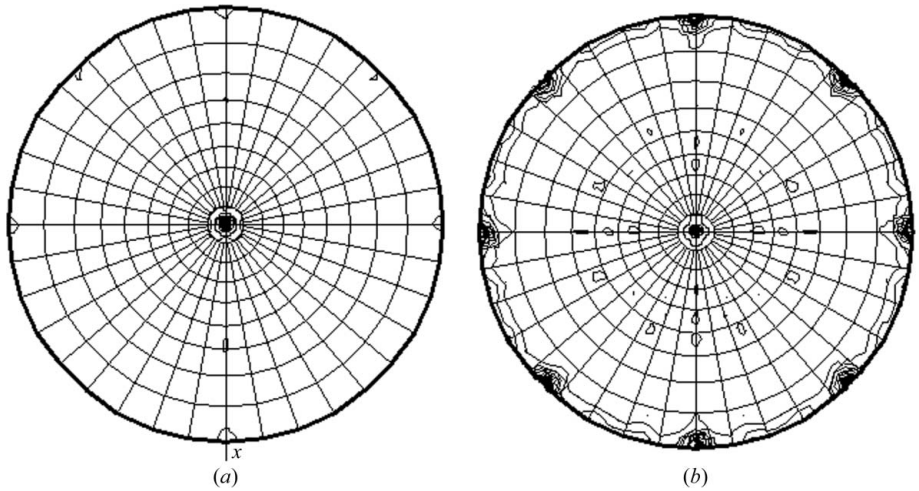


(*a*) (*b*)

Figure 1
(*a*) Crystal form 1 of Pab0087 obtained in 300 m*M* NaCl, 200 m*M* KSCN, 8% PEG 20K, 8% PEG 550 MME pH 8.4. The crystal size was 250 × 150 × 20 µm. (*b*) Crystal form 2 of Pab0087 obtained in 20 m*M* $CaCl_2$, 30% MPD pH 4.6. The crystal size was 200 × 40 × 40 µm.

2.2. Crystallization

The protein was concentrated to 12 mg ml^{-1} in 50 m*M* Tris–HCl buffer pH 8.5, 300 m*M* NaCl using an Amicon Ultra 10K 4 ml (Millipore). Crystals of Pab0087 were grown by vapour diffusion in 2 µl hanging drops comprising equal volumes of protein and reservoir solution at 291 K. After 2–3 d incubation, crystals were observed in two conditions containing 300 m*M* NaCl, 200 m*M* KSCN, 8% PEG



(*a*) (*b*)

Figure 2
Self-rotation function calculated using crystal form 1 data with orthogonalization code 1 (*a*, *c**^*a*, *c**). The integration radius is 25 Å and the resolution limits are 12–3 Å. (*a*) $\chi = 90°$ section. The peak indicates that the fourfold non-crystallographic axis is parallel to *c**. (*b*) $\chi = 180°$ section. This plot shows the twofold crystallographic axis parallel to *b*, the fourfold non-crystallographic axis and three twofold non-crystallographic axis (one parallel to *a* and two orthogonal to *c**).

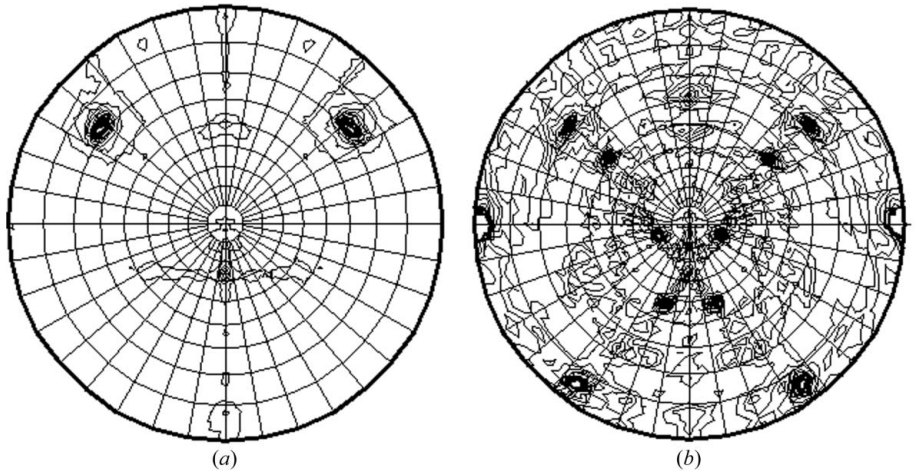


Figure 3
Self-rotation function calculated using crystal form 2 data with orthogonalization code 1 (*a*, *c**^*a*, *c**). The integration radius is 25 Å and the resolution limits are 12–3 Å. (*a*) $\chi = 90^\circ$ section. (*b*) $\chi = 180^\circ$ section.

20K, 8% PEG 550 MME pH 8.4 (solution No. 23, Clear Strategy Screen 1 from Molecular Dimension Ltd) for crystal form 1 and 20 m*M* $CaCl_2$, 30% MPD pH 4.6 (solution No. 1, Crystal Screen from Hampton Research) for crystal form 2. The average dimensions of these crystals were 250 × 150 × 20 µm (Fig. 1*a*) and 200 × 40 × 40 µm (Fig. 2*b*), respectively.

2.3. X-ray diffraction analysis

Crystals were mounted in a cryoloop and flash-frozen in the presence of 30% glycerol for crystal form 1 or directly from the drop for crystal form 2. Diffraction data were collected using a MAR 345 detector on a Micromax 7 rotating-anode generator at the Institut de Biologie et Physico-Chimie, Paris and using a MAR CCD detector at beamline FIP-BM30A of the European Synchrotron Radiation Facility, Grenoble, respectively. The crystal form 1 data set was collected to 2.7 Å resolution with a 360° sweep and that for form 2 was collected to 2.0 Å resolution with a 180° sweep. Data were processed with *XDS* and scaled with *XSCALE* (Kabsch, 1988). Autoindexing and consideration of systematically absent reflections revealed that the two crystal forms belong to the monoclinic space group *C*2, with unit-cell parameters $a = 160.59$, $b = 135.74$, $c = 113.02$ Å, $\beta = 117.36^\circ$ and $a = 166.97$, $b = 131.25$, $c = 189.39$ Å, $\beta = 113.81^\circ$, respectively. Data-collection statistics of the two crystal forms from protein Pab0087 are reported in Table 1.

Using the program *MOLREP* (Vagin, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994), self-rotation functions were computed for each crystal form to test for the presence of non-crystallographic symmetry. A self-rotation function of the form 1 data set indicates the presence of a crystallographic twofold axis, three twofold axes and one perpendicular fourfold axis, suggesting that the protein is a tetramer in the crystal (Figs. 2*a* and 2*b*). Four molecules per asymmetric unit correspond to a Matthews coefficient (Kantardjieff & Rupp, 2003) of 2.71 $Å^3$ Da^{-1} and 53.4% solvent content. Self-rotation functions of the form 2 data set reveal the presence of a crystallographic twofold axis, two fourfold axes and several twofold axes generated by combination of the crystallographic twofold and fourfold axes (Figs. 3*a* and 3*b*). Thus, there are two non-parallel tetramers in the asymmetric unit. Eight molecules per asymmetric unit, totalling 403.2 kDa, correspond to a Matthews coefficient of 2.37 $Å^3$ Da^{-1} and 48.1% solvent content.

3. Conclusion

The Pab0087 protein from *P. abyssi* has been crystallized in two forms. Analysis of the X-ray data from these crystals revealed that Pab0087 crystallized in space group *C*2, with unit-cell parameters $a = 160.59$, $b = 135.74$, $c = 113.02$ Å, $\beta = 117.36^\circ$ for form 1 and $a = 166.97$, $b = 131.25$, $c = 189.39$ Å, $\beta = 113.81^\circ$ for form 2. Calculation of self-rotation functions revealed the presence of one tetramer per asymmetric unit in crystal form 1 and two tetramers in crystal form 2. As the PRPs fold is highly conserved, we are attempting to solve the structure of Pab0087 by molecular replacement using *AMoRe* (Navaza, 1994) with the available structures of similar PRPs as the starting model. In parallel, the production of selenomethionine-substituted protein and a search for heavy-atom derivatives are in progress. We have already used form 2 crystals for soaking in solution containing holmium ($HoCl_3$; Heavy-Atom Screen M2 from Hampton Research). Analyses of the diffraction data from this heavy-atom derivative and other derivatives will allow us to solve the structure by MIR/MAD methods.

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