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Cloning, purification crystallization and preliminary X-ray characterization of a conserved hypothetical protein XC6422 from *Xanthomonas campestris*

Chao-Yu Yang,[a] Ko-Hsin Chin,[a] Chia-Cheng Chou,[b,c] Hui-Lin Shr,[b,c] Fei Philip Gao,[d] Ping-Chiang Lyu,[e] Andrew H.-J. Wang[b,c] and Shan-Ho Chou[a]*

[a]Institute of Biochemistry, National Chung-Hsing University, Taichung 40227, Taiwan, [b]Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei, Taiwan, [c]Core Facility for Protein Crystallography, Academia Sinica, Nankang, Taipei, Taiwan, [d]National High Magnetic Field Laboratory, Florida State University, Tallahassee, FL 32310, USA, and [e]Department of Life Science, National Tsing Hua University, Hsin-Chu, Taiwan

Correspondence e-mail: shchou@nchu.edu.tw

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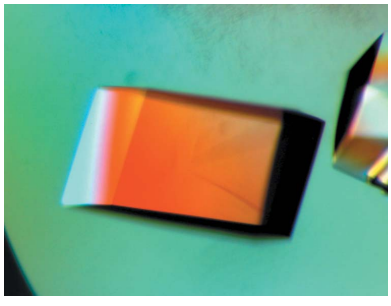


Xanthomonas campestris pv. *campestris* is a Gram-negative yellow-pigmented pathogenic bacterium that causes black rot, one of the major worldwide diseases of cruciferous crops. Its genome contains approximately 4500 genes, roughly one third of which have no known structure and/or function. However, some genes of unknown function are highly conserved among several different bacterial genuses. XC6422 is one such conserved hypothetical protein and has been overexpressed in *Escherichia coli*, purified and crystallized in a variety of forms using the hanging-drop vapour-diffusion method. Crystals grew to approximately 2 × 1.5 × 0.4 mm in size after one week and diffracted to at least 1.6 Å resolution. They belong to the monoclinic space group *C*2, with one molecule per asymmetric unit and unit-cell parameters a = 75.8, b = 79.3, c = 38.2 Å, β = 109.4°. Determination of this structure may provide insights into the protein's function.

1. Introduction

Structural genomics is a new and rapidly developing field in biology (Edwards *et al.*, 2004; Zhang & Kim, 2004) and has been under active investigation worldwide. The goal of this frontier research is to discover novel protein folds and to solve the structures of a representative sample of protein molecules in order to obtain a more thorough understanding of biology from a structural perspective. We have joined the efforts and initiated a structural genomics program of the local plant pathogen *Xanthomonas campestris* pv. *campestris* strain 17 (Xcc). We have focused on studying its unique regulatory pathway towards pathogenicity. Xcc Clp (cAMP-receptor like protein) has been found to be a global transcription factor that is homologous to the cAMP receptor of *Escherichia coli*. It forms a complex with cAMP and is involved in the regulation of over 100 important cellular functions in *Enterobacteriaceae* and *Bacillus* (Ebright, 1993; Kolb *et al.*, 1993). In Xcc, however, no cAMP-mediated signal transduction pathway was discovered and Clp was found to be solely responsible for regulating a wide variety of genes necessary for the synthesis of exopolyaccharides, extracellular enzyme and components of the apparatus for type II protein secretion, all of which are collectively required for its pathogenicity (de Crecy-Lagard *et al.*, 1990; Chen & Tseng, 2005). Xcc is thus a peculiar phytopathogen that deserves more thorough studies in structural terms.

XC6422 has been classified as a conserved hypothetical protein by a bioinformatics approach (http://xcc.life.nthu.edu.tw/). It contains 220 amino acids and shares 94% sequence identity with a similar protein from the *Xanthomonas* genus (*X. campestris* pv. *campestris* strain ATCC 33913; gi|21111227; da Silva *et al.*, 2002), 71% identity with a protein from *Xylella fastidiosa* (gi|9106911; Simpson *et al.*, 2000) and 38% identity with a protein from *Ralstonia solanacearum* (gi|17427337; Salanoubat *et al.*, 2002). To date, no homologous structure for the XC6422 protein has been reported in the PDB, although it is classified as a putative hydrolase belonging to the α/β-superfamily in the COG database (Tatusov *et al.*, 2001). In this report, we describe the cloning, purification, crystallization and initial X-ray analysis of XC6422.

2. Materials and methods

2.1. Cloning, expression and purification

The XC6422 gene fragment was PCR-amplified directly from a local Xcc genome (*X. campestris* pv. *campestris* strain 17) with a forward 5′-TACTTCCAATCCAATGCTA*TGTCCAATCCCTTAT-TCCCCACC* primer and a backward 5′-TTATCCACTTCCAATG*T-CAGGGCGTGGCCGGTAGCCAG* primer. A ligation-independent cloning (LIC) approach (Aslanidis & de Jong, 1990) was carried out to obtain the desired construct. A pTBSG1 vector (F. P. Gao, unpublished results) was cut to completion with *Ssp*I (Novagen). For LIC, 100 ng of the linearized vector and 100 ng of the PCR product were treated with 2 units of T4 DNA polymerase (Novagen) in separate reactions in the presence of 2.5 m*M* dGTP or dCTP and 5 m*M* DTT, respectively. The reactions were carried out at 298 K for 30 min. The enzyme was subsequently heat-inactivated at 348 K for 20 min. The vector and the PCR product were then mixed and heated at 301 K for 3 min before cooling to room temperature. The mixture was directly transformed into the *E. coli* BL21 (DE3) host without ligation. The final construct codes for an N-terminal His_6 tag, a 17-amino-acid linker and an XC6422 protein (220 amino acids) under the control of a T7 promoter. Transformed *E. coli* BL21 (DE3) host cells were grown in LB medium at 310 K until an OD_{600} of 0.8 was attained. Overexpression was induced by the addition of 1 m*M* IPTG at 310 K for 3.5 h. The cells were harvested, resuspended in equilibration buffer (20 m*M* Na_2HPO_4, 70 m*M* NaCl pH 8.0) and lysed using a microfluidizer (Microfluidics). Most tagged target proteins were in the soluble fraction (Fig. 1). After centrifugation, the target protein was purified by immobilized metal-affinity chromatography (IMAC) on a nickel column (Sigma), which was eluted with 20 m*M* Tris pH 8.0, 70 m*M* NaCl and a gradient of 50–300 m*M* imidazole. The fractions containing XC6422 were monitored by SDS–PAGE and recombined and dialyzed repeatedly against 50 m*M* Na_2HPO_4 pH 8.0, 10% glycerol and 500 m*M* NaCl. After buffer exchange, the His_6 tag was cleaved from XC6422 by TEV (tobacco etch virus) protease at 283 K for 12 h to obtain the cleaved product. Upon rechromatography on the nickel column, the target XC6422 protein was in the flowthrough fractions, with the His_6 tag and the tagged TEV protease retained on the nickel column. The purified protein was then dialyzed against 20 m*M* Tris pH 8.0 and 70 m*M* NaCl. For crystallization, XC6422 was further purified on an anion-exchange column (AKTA, Pharmacia Inc.). The fractions eluted with 20 m*M* Tris pH 8.0, 750 m*M* NaCl were combined and dialyzed against 20 m*M* Tris pH 8.0 and 70 m*M* NaCl. The final target protein (220 amino acids) has greater than 99% purity (Fig. 1) and contains only an extra tripeptide (SNA) at the N-terminal end with an expected MW of 24 251 Da, which was confirmed by mass-spectrometric analysis. The overexpression and purification of XC6422 was monitored by SDS–PAGE as shown in Fig. 1.

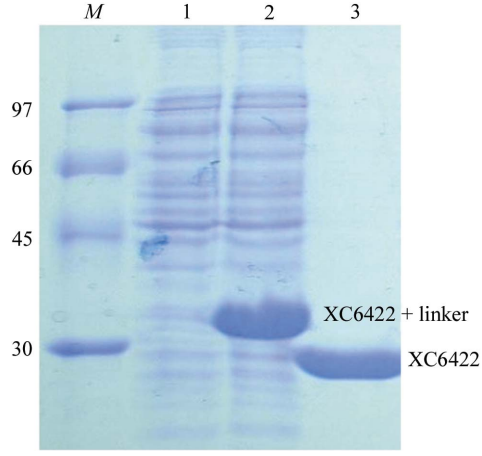


Figure 1
SDS–PAGE monitoring of the overexpression and purification of XC6422. Lane *M*, molecular-weight markers in kDa; lane 1, soluble fraction before IPTG induction; lane 2, soluble fraction after IPTG induction; lane 3, purified XC6422 after TEV cleavage. The positions of fused and free XC6422 are also marked.

Table 1
Data-collection statistics for native XC6422 crystals.

Values in parentheses are for the highest resolution shell.

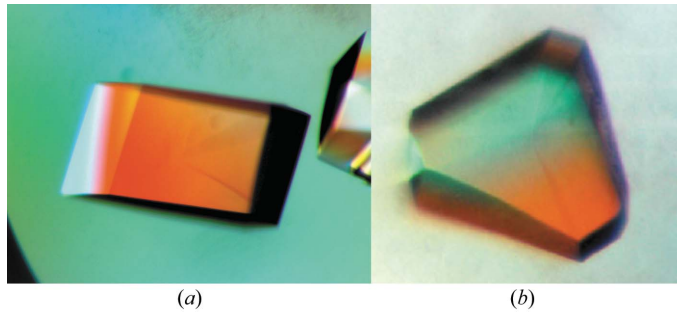
Space group	*C*2
Unit-cell parameters (Å, °)	a = 75.8, b = 79.3, c = 38.2, β = 109.4
Data-collection temperature (K)	100
Wavelength (Å)	1.5418
Resolution range (Å)	24.8–1.60 (1.66–1.60)
Mosaicity (°)	0.3
Unique reflections	27824 (2547)
Redundancy	2.9 (2.4)
Completeness (%)	99.1 (91.3)
R_{merge} (%)	4.1 (14.4)
Mean $I/\sigma(I)$	14.7 (4.6)
Solvent content (%)	45.12

2.2. Crystallization

For crystallization, the protein was concentrated to 35.5 mg ml^{-1} in 20 m*M* Tris pH 8.0 and 70 m*M* NaCl using an Amicon Ultra-10 (Millipore). Crystallization screening was performed using sitting-drop vapour diffusion in 96-well plates (Hampton Research) at 295 K by mixing 0.5 µl protein solution with 0.5 µl reagent solution. Initial screens included the Hampton sparse-matrix Crystal Screens 1 and 2, a systematic PEG–pH screen and the PEG/Ion Screen and were performed using a Gilson C240 crystallization workstation. Parallelepiped-shaped and prism-shaped crystals appeared in 1 d from a reservoir solution comprising 0.1 *M* HEPES buffer pH 7.5, 2.0 *M* $(NH_4)_2SO_4$ and 2% PEG 400. This initial condition was then optimized by varying the concentration of ammonium sulfate. Crystals suitable for diffraction experiments were grown by mixing 1.5 µl protein solution with 1.5 µl reagent solution and reached maximum dimensions of 2.0 × 1.5 × 0.4 mm after one week (Fig. 2).

2.3. Data collection

Crystals were soaked in a cryoprotectant solution comprising reservoir solution plus 20%(*v*/*v*) glycerol and were then flash-cooled at 100 K in a stream of cold nitrogen. X-ray diffraction data were



(*a*) (*b*)

Figure 2
Two different crystal forms of XC6422 grown by the sitting-drop vapour-diffusion method. Crystallization conditions were optimized to 0.1 *M* HEPES buffer pH 7.5, 1.6 *M* $(NH_4)_2SO_4$ and 2% PEG 400 (*a*) and 0.1 *M* Tris buffer pH 8.5, 2.2 *M* $(NH_4)_2SO_4$ and 2% PEG 400 (*b*). The approximate dimensions of these crystals were 2 × 1.5 × 0.4 mm.

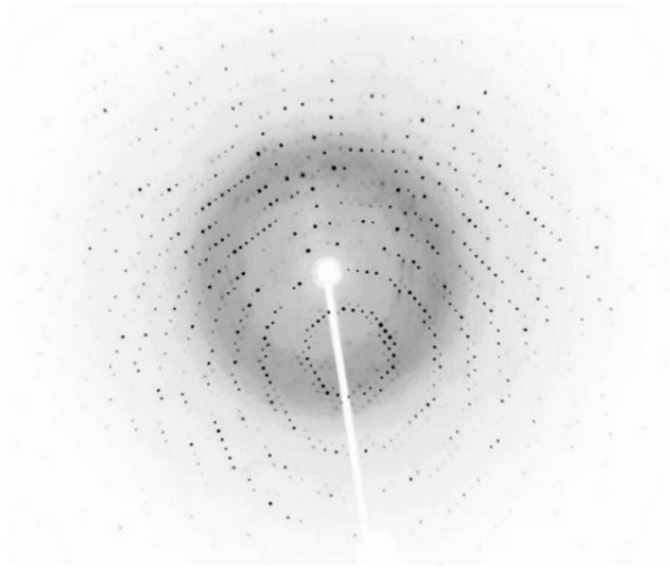


Figure 3
Diffraction pattern of XC6422 collected in-house from a crystal flash-frozen with 20% glycerol cryoprotectant. The exposure time was 10 min, with an oscillation range of 1.0° and a crystal-to-detector distance of 100 mm.

collected using Cu $K\alpha$ radiation from a Rigaku RU-300 rotating-anode generator equipped with Osmic mirror optics and an R-AXIS IV^{++} image plate. A native data set was obtained to a resolution of at least 1.6 Å; the data were indexed and integrated using the *HKL* software suite (Otwinowski & Minor, 1997), giving a data set that was 99.1% complete (91.3% in the last shell) with an overall $R_{\rm merge}$ of 4.1% on intensities. The crystals belong to the monoclinic space group *C*2, with one molecule in each asymmetric unit, and contain 45.1% solvent. The data-collection statistics are summarized in Table 1. An X-ray diffraction image collected in-house is shown in Fig. 3.

3. Results and discussion

The gene sequence of XC6422 was confirmed after cloning and consists of 663 bp coding for a protein of 220 amino-acid residues, with a calculated isoelectric point of 4.83. The purified XC6422 showed a single band of approximately 24 kDa on SDS–PAGE, with a greater than 99% purity (Fig. 1). Such a high purity possibly accounts for its straightforward crystallization; good crystals of up to 2.0 × 1.5 × 0.4 mm in size with high diffraction resolution of at least 1.60 Å were readily obtained in one week (Fig. 2).

We have chosen proteins with unknown structure and/or unknown functions as our targets to increase the possibility of discovering novel protein folds. High-resolution diffraction data were obtained for the native XC6422 crystals (one of which is shown in Fig. 3), indicating their suitability for further detailed X-ray structural analysis. We now plan to solve the structure of XC6422 using either the multiple isomorphous replacement (MIR) method (Ke, 1997) or the multiwavelength anomalous diffraction (MAD) method using selenomethionine-substituted protein (Hendrickson & Ogata, 1997), given that XC6422 contains one cysteine and four methionines, respectively. Heavy-atom positions and phases will be solved using automated Patterson analysis as described in Terwilliger & Berendzen (1999).

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