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Cloning, expression, crystallization and preliminary X-ray analysis of a putative multiple antibiotic resistance repressor protein (MarR) from *Xanthomonas campestris*

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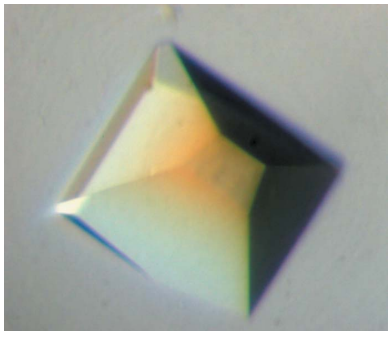


The multiple antibiotic resistance operon (*marRAB*) is a member of the multidrug-resistance system. When induced, this operon enhances resistance of bacteria to a variety of medically important antibiotics, causing a serious global health problem. MarR is a *marR*-encoded protein that represses the transcription of the *marRAB* operon. Through binding with salicylate and certain antibiotics, however, MarR can derepress and activate the *marRAB* operon. In this report, the cloning, expression, crystallization and preliminary X-ray analysis of XC1739, a putative MarR repressor protein present in the *Xanthomonas campestris* pv. *campestris*, a Gram-negative bacterium causing major worldwide disease of cruciferous crops, are described. The XC1739 crystals diffracted to a resolution of at least 1.8 Å. They are orthorhombic and belong to space group $P2_12_12_1$, with unit-cell parameters a = 39.5, b = 54.2 and c = 139.5 Å, respectively. They contain two molecules in the asymmetric unit from calculation of the self-rotation function.

1. Introduction

The emergence of multiply antibiotic resistant bacteria is proceeding at an alarming rate. Many antibiotics are becoming gradually ineffective in combating infectious diseases because pathogenic strains are developing multiple antibiotic resistance (*mar*) phenotypes. In contrast to multiple antibiotic resistance mediated by plasmids or tranposons (Foster, 1983), chromosomal multiple antibiotic resistance has also been detected (George, 1996), with the *marRAB* operon of *Escherichia coli* as the prototype (George & Levy, 1983). Such a chromosomal *mar* family controls resistance not only to antibiotics, but also to organic solvents, detergents, toxic chemicals and oxidative stress agents (Alekshun & Levy, 1997, 1999). Three proteins are encoded by the *marRAB* operon: MarR (a repressor), MarA (a transcriptional activator) and MarB (a protein of unknown function; Alekshun & Levy, 1999). In the absence of an inducer, MarR binds to *marO* and negatively regulates transcription of *marRAB* (Martin & Rosner, 1995). A large number of compounds with aromatic rings such as salicylate, tetracycline, chloramphenicol *etc*. have been found to induce the transcription of *marRAB* operon by binding tightly to MarR, with the resulting complexes failing to bind to the promoter DNA (Cohen *et al.*, 1993). When the MarR repression is missing, transcription of the operon then ensues.

XC1739 from the plant pathogen *Xanthomonas campestris* pv. *campestris* strain 17 (Xcc) is classified as belonging to the MarR family in the Pfam database (Bateman *et al.*, 2000). It contains 162 amino acids and shares 80% identity with a similar protein in the *Xylella fastidiosa* genome (Dow & Daniels, 2000), 25% identity with a protein in the *Ralstonia solanacearum* genome (Salanoubat *et al.*, 2002), 100% identity with a similar protein in the *X. campestris* pv. *campestris* ATCC33913 genome (gi|21111420; da Silva *et al.*, 2002), 32% identity (55.6% similarity) with a MarR protein in the *E. coli* genome (Alekshun *et al.*, 2001) and 22.6% identity (66.0% similarity) with a MexR protein in the *Pseudomonas aeruginosa* genome (Lim *et al.*, 2002). Recently, a complex of MarR with its inducer salicylate has been co-crystallized and its structure determined to a resolution of 2.3 Å by the X-ray diffraction method (Alekshun *et al.*, 2001). However, the MarR crystals grown in the absence of salicylate were

found to be badly disordered, precluding crystallographic characterization of MarR in the absence of an inducer (Alekshun *et al.*, 2001). In this manuscript, we describe the cloning, purification, crystallization and initial X-ray analysis of a putative MarR protein (XC1739) from *X. campestri* without the salicylate ligand.

2. Materials and methods

2.1. Cloning, expression and purification

The XC1739 gene fragment was PCR-amplified directly from a local Xcc genome (*X. campestris* pv. *campestris* strain 17). It was cut with the *Ssp*I restriction enzyme and cloned into a ligation-independent vector pTBSG1 (F. P. Gao, unpublished results). The final construct codes for an N-terminal His_6 tag, a 17-amino-acid linker and an XC1739 protein (162 amino acids) under the control of a T7 promoter. The transformed *E. coli* BL21 (DE3) host cell was grown in LB medium at 310 K until an OD_{600} of 0.8 was attained. Overexpression of the His_6-tagged protein was induced by the addition of 1 m*M* IPTG at 310 K for 4 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 XC1739 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 XC1739 using TEV (tobacco etch virus) protease at 283 K for 12 h to obtain the cleaved product. Without the His_6 tag, the target XC1739 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 XC1739 was further purified on a cation-exchange column (AKTA, Pharmacia Inc.). The fractions eluted with 20 m*M* Tris pH 8.0, 400 m*M* NaCl were combined and dialyzed against 20 m*M* Tris pH 8.0 and 70 m*M* NaCl. The final target protein (162 amino acids) has a greater than 99% purity (Fig. 1) and only contains an extra tripeptide (SNA) at the N-terminal end. The overexpression and purification of XC1739 was monitored by SDS–PAGE and is shown in Fig. 1.

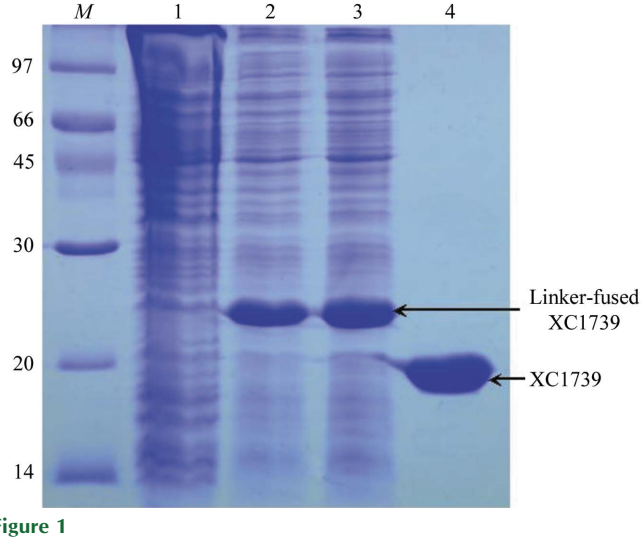


Figure 1
SDS–PAGE monitoring of the overexpression and purification of XC1739. Lane *M*, molecular-weight markers in kDa; lane 1, whole cell lysate before IPTG induction; lane 2, whole cell lysate after IPTG induction; lane 3, soluble fraction after IPTG induction; lane 4, purified XC1739 after TEV cleavage. The positions of linker-fused and free XC1739 are also marked.

Table 1
Data-collection statistics for XC1739.

Values in parentheses are for the highest resolution shell.

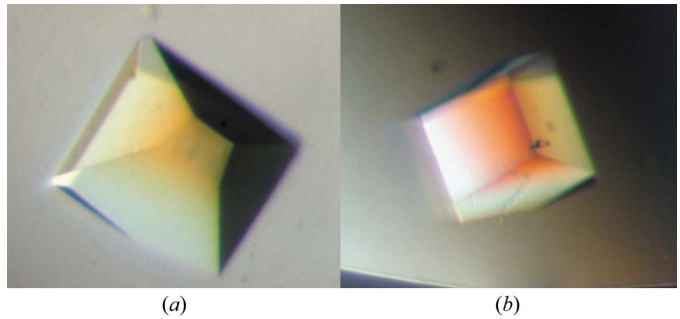
Space group	$P2_12_12_1$
Unit-cell parameters (Å)	a = 39.5, b = 54.2, c = 139.5
Temperature (K)	100
Wavelength (Å)	1.5418
Resolution range (Å)	46.6–1.8 (2.38–1.80)
Mosaicity (°)	0.4
Unique reflections	13837 (1236)
Redundancy	4.0 (2.8)
Completeness (%)	98.2 (90.0)
R_{merge} (%)	3.6 (8.0)
Mean $I/\sigma(I)$	21.5 (13.2)
Solvent content (%)	67.9 or 35.9

2.2. Crystallization

For crystallization, the protein was concentrated to 22 mg ml^{-1} in 20 m*M* Tris pH 8.0 and 70 m*M* NaCl using an Amicon Ultra-10 (Millipore). Screening for crystallization conditions 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 the Gilson C240 crystallization workstation. Pyramid-shaped crystals appeared in 2 d from a reservoir solution comprising 0.1 *M* sodium acetate buffer pH 5.5, 0.2 *M* $MgCl_2$ and 25% PEG 2K MME (polyethylene glycol monomethyl ether). This initial condition was then optimized by varying the concentrations of $MgCl_2$ and PEG, the optimum concentrations of which were found to be 0.2 *M* and 22%, respectively. Pyramid-shaped 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 0.1 × 0.1 × 0.15 mm after 3 d (Fig. 2).

2.3. Data collection

Crystals were soaked in mother liquor and then flash-cooled at 100 K in a stream of cold nitrogen. X-ray diffraction data were collected using Cu $K\alpha$ radiation from a Rigaku MicroMax007 rotating-anode generator equipped with Osmic mirror optics and an R-AXIS IV^{++} image plate. A 1.8 Å resolution native data set was



(*a*) (*b*)

Figure 2
Pyramid-shaped crystals of MarR from *X. campestris* grown by the sitting-drop vapour-diffusion method. The crystallization conditions used were 0.1 *M* sodium acetate buffer pH 5.5, 0.2 *M* $MgCl_2$ and 22% PEG 2K MME (*a*). Crystallization was also attempted by microseeding microscopic crystal fragments into the same protein solution to increase the crystal volume. Different crystal forms were observed (*b*). The average dimensions of these crystals were all around 0.1 × 0.1 × 0.2 mm.

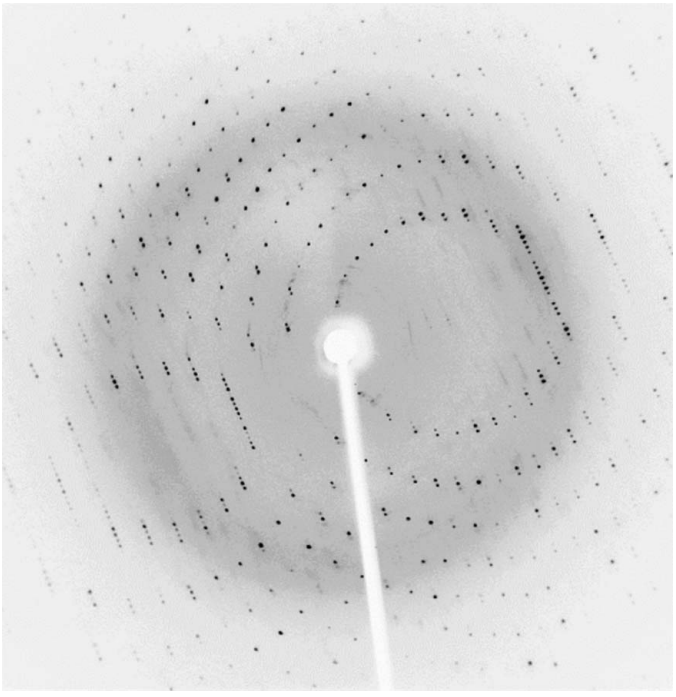


Figure 3
Diffraction pattern collected in-house from a flash-frozen crystal in mother liquor as cryoprotectant. The exposure time was 12 min, with an oscillation range of 1.0° and a crystal-to-detector distance of 180 mm.

obtained. Because the lack of a 2θ stage, the crystal-to-detector distance was moved to 180 mm to ensure that all diffraction spots are well separated. A better data set will be collected at a synchrotron facility. The data were indexed and integrated using *HKL* processing software (Otwinowski & Minor, 1997), giving a data set that had 98.2% completeness and an overall $R_{\rm merge}$ of 3.6% on intensities. The crystals belong to the orthorhombic space group $P2_12_12_1$, with significant systematic absences of diffraction spots along the (h00), (0k0) and (00l) directions. Two molecules per asymmetric unit were revealed from the non-crystallographic twofold symmetry calculated from the self-rotation function map containing data in the resolution range 25–5.0 Å (data not shown). The data-collection statistics are summarized in Table 1, with an X-ray diffraction image collected in-house shown in Fig. 3.

3. Results and discussion

The gene of XC1739 consists of 486 bp coding for 162 amino-acid residues. The isoelectric point was calculated to be 8.05. The purified XC1739 showed a single band of approximately 18 kDa on SDS–PAGE (Fig. 1), which is in good agreement with the calculated value of 18.2 kDa.

Although the sequence identities of XC1739 (gi|21111420) with the MarR of *E. coli* and MexR of *P. aeruginosa* are in the grey area (32 and 22.6%, respectively), the similarities are very high (55.6 and 66.0%, respectively). Furthermore, the predicted secondary-structure topology of XC1739 (α1-α2-β1-α3-α4-β2-β3-α5-α6) is very similar to those of *E. coli* MarR (Alekshun *et al.*, 2001) and *P. aeruginosa* MexR (Lim *et al.*, 2002), with all conserved residues mapped to the similar N- and C-terminal domains and DNA-binding domains (Alekshun *et al.*, 2001). The above arguments indicate that *X. campestris* XC1739 is very likely to be a MarR protein and is the first report of the existence of a possible *mar* regulon in a plant pathogen.

The high-resolution diffraction obtained from the native crystals establishes its suitability for X-ray structural analysis (Fig. 3). Although the three-dimensional structure of a MarR–salicylate complex from *E. coli* has been determined by the X-ray method (Alekshun *et al.*, 2001), so far the MarR structure in its unliganded state has not been determined, although the structure of MexR, a MarR analogue, has been determined without a bound ligand (Lim *et al.*, 2002). We are therefore engaged in determining the native MarR protein of XC1739 and investigating the conformational differences between the liganded and unliganded states. However, it is worth mentioning that even though we did not explicitly add salicylate during the crystallization process, it is still possible that an unexpected ligand has accidentally copurified with the protein during the preparation stages (Frenois *et al.*, 2004). Hence, the claim that the crystals we obtained are in the unliganded state needs to be substantiated until the final structure has been determined. Since XC1739 contains seven methionines, we now plan to solve its structure by the multiwavelength anomalous diffraction (MAD) method using selenomethionine-substituted protein (Hendrickson & Ogata, 1997; Terwilliger & Berendzen, 1999).

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