Purification, crystallization and X-ray crystallographic studies of a *Bacillus cereus* MepR-like transcription factor, BC0657

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Transcription factors of the MarR family respond to internal and external changes and regulate a variety of biological functions through ligand association with microorganisms. MepR belongs to the MarR family, and its mutations are associated with the development of multidrug resistance in *Staphylococcus aureus*, which has caused a growing health problem. In this study, a *Bacillus cereus* MepR-like transcription regulator, BC0657, was crystallized. The BC0657 crystals diffracted to 2.05 Å resolution and belonged to either space group $P6_22$ or $P6_422$, with unit-cell parameters $a = 110.57$, $b = 110.57$, $c = 67.29$ Å. There was one molecule per asymmetric unit. Future comparative structural studies on BC0657 would extend knowledge of ligand-induced transcriptional regulatory mechanisms in the MarR family and would make a significant contribution to the design of antibiotic drugs against multidrug-resistant bacteria.

1. Introduction

Microorganisms possess defence mechanisms in which the products of regulatory genes recognize the presence of external or internal danger signals and control the expression of defence-related genes at the transcriptional level. In *Escherichia coli*, a multiple antibiotic-resistance regulator (MarR) protein negatively regulates gene expression in the *marRAB* operon that manifests the Mar phenotype characterized as multi-drug resistance (Cohen *et al.*, 1993). A number of bacterial and archaeal MarR-homologous proteins have been identified to date and have been shown to regulate the transcription of genes involved in stress response, virulence and degradation or export of harmful chemicals such as phenolic compounds, antibiotics and detergents (Aleksun & Levy, 1997). The MarR family proteins function as triangular-shaped dimers that symmetrically associate with palindromic operator DNA within their cognate promoters using two winged helix–turn–helix (wHTH) domains (Aleksun *et al.*, 2001; Hong *et al.*, 2005). However, each MarR protein has evolved to utilize a unique ligand-recognition mechanism for transcriptional regulation, given that MarR proteins interact with their ligands using one to eight structurally distinct sites (Davis *et al.*, 2013; Saridakis *et al.*, 2008; Chang *et al.*, 2010; Aleksun *et al.*, 2001).

Although MarR-mediated defence mechanisms are beneficial to the survival and growth of microbial pathogens, bacterial adaptation to antimicrobial drugs results in multi-drug-resistant pathogens and may be harmful or even fatal to infected hosts. For example, mutations in the multidrug efflux pump regulator (*mepR*), a *marR*-homologous gene, led to the

Table 1

Macromolecule-production information.

Source organism	<i>B. cereus</i> ATCC 14579
DNA source	Genomic DNA
Forward primer†	GAAGGATCCGGGACCAATGATATGACGCATATC- GAT
Reverse primer†	CTTGTCGACTTAGTTATTCTCTTTTCTCTGT- GTC
Cloning vector	pET-49bm
Expression vector	pET-49bm
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	MPNDMTHIDKIQLAFSIGKKMQTELEQM QATG- LTPPQFYILKILDHYGASRATTLAKKMYVKPS- AITVMIDRLIDQELVERYHDKDDRRVVI IELT- KKGKARVEEAMTARNEHI AKYFSHLELQERED- LLRLF EKLETTICGTQ EKKENN

† BamHI and SalI restriction-enzyme sites are underlined.

development of multidrug-resistant strains of *Staphylococcus aureus* in hospitals (Kaatz *et al.*, 2005, 2006). MepR inhibits the expression of MepA, a multidrug efflux pump that confers resistance to a wide range of toxic compounds, including antimicrobial fluoroquinolones and biocides. MepR binds the promoter region of the *mepA* gene and prevents RNA polymerase from transcriptional initiation at the *mepA* gene. Recently, MepR mutants that were not able to repress *mepA* transcription were identified in clinical *S. aureus* strains that overexpress MepA. As a result, the clinical strains that harbour MepR mutations could not be controlled by typical antibiotics and led to severe medical problems (Schindler *et al.*, 2013). Structure-based functional analyses of MepR mutants as well as MepR in complex with DNA demonstrated that the mutations reconfigure MepR into a structure with impaired DNA binding and cause constitutive expression of *mepA* (Birukou *et al.*, 2013, 2014). However, owing to the absence of the structure of MepR complexed with ligand molecules, it remains unknown how the MepR virulence factor of pathogenic bacteria recognizes ligand molecules and triggers ligand-mediated regulation of MepA expression. Additionally, a structural study on the MepR-ligand complex is required to provide deeper insight into the structure-based design of novel antibacterial drugs susceptible to multidrug-resistant strains. Here, as a first step towards structural analysis of the MepR-ligand association, we report the expression, purification and X-ray diffraction studies of a MepR-like transcription factor, BC0657, from the pathogenic bacterial species *Bacillus cereus*.

2. Materials and methods

2.1. Macromolecule production of recombinant BC0657

Genomic DNA of *B. cereus* was obtained using the Exgene Cell SV kit (GeneAll). The BC0657 gene (residues 1–152) was amplified by PCR from the genomic DNA of *B. cereus* using forward and reverse primers. The amplified BC0657 gene was treated sequentially with BamHI and SalI and ligated into a modified pET-49b vector (pET-49bm). The pET-49bm vector contains an N-terminal His₆ tag and thrombin cleavage site for purification in affinity chromatography and removal of the

Table 2

Crystallization.

Method	Sitting-drop vapour diffusion
Plate type	Cryschem plates
Temperature (K)	291
Protein concentration (mg ml ⁻¹)	34
Buffer composition of protein solution	20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM βME
Composition of reservoir solution	0.1 M phosphate-citrate buffer pH 4.0, 1.8 M ammonium sulfate
Volume and ratio of drop	0.5 µl + 0.5 µl
Volume of reservoir (µl)	500

affinity tag, respectively. The resulting ligation product was transformed into *E. coli* DH5α cells. The nucleotide sequence of the BC0657 expression plasmid was confirmed by DNA sequencing.

The BC0657 expression plasmid was transformed into *E. coli* strain BL21 (DE3) for protein expression. Cells were grown at 37°C with vigorous shaking in LB medium containing 100 µg ml⁻¹ kanamycin. After the OD₆₀₀ (the optical density

(a)

(b)

Figure 1

Purification and crystallization of BC0657. (a) SDS-PAGE analysis of the purified BC0657 protein (lane 2) and protein standards (lane 1; labelled in kDa). (b) The BC0657 crystals (~0.12 × ~0.05 × ~0.05 mm) used for X-ray diffraction.

at 600 nm) had reached ~ 0.7 , the recombinant BC0657 protein was overexpressed at 18°C for ~ 16 h in the presence of 1 mM isopropyl β -D-1-thiogalactopyranoside.

The cells were harvested by centrifugation. The cell pellet was resuspended in phosphate-buffered saline (PBS) pH 7.4 supplemented with 10 mM imidazole and 5 mM β -mercaptoethanol (β ME). The cells were lysed by sonication and the lysate was cleared by centrifugation ($\sim 25\,000g$). The supernatant was incubated with Ni-NTA agarose resin (Qiagen) at 4°C for 3 h. The resin was collected and washed in an Econo-Column (Bio-Rad) using PBS supplemented with 10 mM imidazole and 5 mM β ME. The bound BC0657 protein was eluted with PBS supplemented with 250 mM imidazole and 5 mM β ME and dialyzed against 20 mM HEPES pH 7.4, 150 mM NaCl, 5 mM β ME. The His₆ affinity tag was removed by thrombin (EMD Millipore) at 4°C for ~ 16 h. The BC0657 protein in the dialysis buffer was further purified by gel-filtration chromatography using a Superdex 200 16/60 column. The fractions that contained BC0657 were concentrated to $\sim 34\text{ mg ml}^{-1}$ for crystallization screening and optimization. BC0657 production information is summarized in Table 1.

2.2. BC0657 crystallization

Crystallization screening was carried out by the sitting-drop vapour-diffusion method using The JCSG Core Suite kit (Qiagen) in MRC 96-well plates (Molecular Dimensions). 0.5 μ l BC0657 protein at $\sim 34\text{ mg ml}^{-1}$ was mixed with 0.5 μ l well solution in 96-well crystallization plates for crystallization screening. BC0657 crystals were initially obtained in 2.0 M ammonium sulfate, 0.1 M phosphate–citrate buffer, which was prepared by mixing 0.2 M dibasic sodium phosphate and 0.2 M

citric acid at the desired pH of 4.0–4.4. The crystallization condition was optimized using 24-well Cryschem plates in ammonium sulfate solutions that contained phosphate–citrate buffer at 18°C. Diffraction-quality crystals were obtained in 0.1 M phosphate–citrate buffer pH 4.0, 1.8 M ammonium sulfate. Crystallization information is summarized in Table 2.

2.3. Data collection and processing

For cryoprotection, BC0657 crystals were directly transferred into and briefly soaked in a solution consisting of 0.1 M phosphate–citrate buffer pH 4.0, 1.8 M ammonium sulfate, 30% glycerol. A single crystal was flash-cooled in a liquid-nitrogen cryostream and was maintained at 100 K during data collection. A total of 240 frames of X-ray diffraction images were collected with an oscillation of 0.5° , an exposure time of 1 s per image and a crystal-to-detector distance of 290 mm using an ADSC Q315r detector on beamline 5C of the Pohang Accelerator Laboratory (PAL), Republic of Korea. Intensity data from the BC0657 crystal were indexed, integrated and scaled using the *HKL-2000* package (Otwinowski & Minor, 1997). Data-processing statistics are summarized in Table 3.

3. Results and discussion

B. cereus BC0657 was overexpressed as an N-terminally His₆-tagged protein in a bacterial expression system using *E. coli* BL21 (DE3) cells. The recombinant BC0657 protein was purified by Ni-NTA affinity chromatography, digested with thrombin and further purified by gel-filtration chromatography, with a protein yield of ~ 3 mg from 1 l of culture. The protein band for BC0657 (calculated molecular weight

Figure 2

An X-ray diffraction image of the BC0657 crystal collected on beamline 5C at PAL. The edge of the frame is at 1.53 Å resolution. The box shows diffraction spots in the outer resolution shell.

Table 3

Data collection and processing.

Values in parentheses are for the outer shell.

Diffraction source	Beamline 5C, PAL
Wavelength (Å)	0.9796
Temperature (K)	100
Detector	ADSC Q315r
Crystal-to-detector distance (mm)	290
Rotation range per image (°)	0.5
Total rotation range (°)	120
Exposure time per image (s)	1.0
Space group	<i>P</i> ₆ ₂ ₂ or <i>P</i> ₆ ₄ ₂ ₂
<i>a</i> , <i>b</i> , <i>c</i> (Å)	110.57, 110.57, 67.29
α , β , γ (°)	90, 90, 120
Mosaicity (°)	0.48
Resolution range (Å)	50.00–2.05 (2.09–2.05)
Total No. of reflections	189949
No. of unique reflections	15507
Completeness (%)	98.6 (100.0)
Multiplicity	12.3 (12.3)
$\langle I/\sigma(I) \rangle$	12.1 (7.9)
$R_{\text{meas}}^{\dagger}$ (%)	5.5 (45.6)
$R_{\text{r.i.m.}}^{\ddagger}$ (%)	5.5 (13.1)
$CC_{1/2}$	0.99 (0.99)
Overall <i>B</i> factor from Wilson plot (Å ²)	36.67

$\dagger R_{\text{meas}}$ was estimated by multiplying the conventional R_{merge} value by the factor $[N/(N-1)]^{1/2}$, where N is the data multiplicity. $\ddagger R_{\text{r.i.m.}} = \sum_{hkl} \{N(hkl)/[N(hkl)-1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

17.7 kDa) was located between the 15 and 20 kDa protein standards in SDS–PAGE analysis and was estimated to be highly homogeneous, with a purity of at least 97% (Fig. 1a).

BC0657 crystals were obtained in ammonium sulfate solutions buffered in dibasic sodium phosphate and citric acid at pH 4.0–4.4. Crystals grew to a visible size of approximately ~20 µm over 8 h and completed their growth to approximately 100 µm in size in 3 d (Fig. 1b). Single BC0657 crystals diffracted to 2.05 Å resolution (Fig. 2). All intensity data were processed using *HKL*-2000, and a summary of the data-processing statistics is shown in Table 2. Indexing and systematic absences of reflections at the screw axis along *c* indicated that the BC0657 crystal belonged to the primitive hexagonal space group *P*₆₂₂ or *P*₆₄₂₂, with unit-cell parameters *a* = 48.87, *b* = 76.47, *c* = 100.67 Å. The Matthews coefficient and the solvent content were calculated to be 3.35 Å³ Da^{−1} and 63%, respectively, suggesting that there is one BC0657 molecule in the asymmetric unit (Matthews, 1968;

Kantardjieff & Rupp, 2003). The crystal structure and the functional implications of the MepR-like BC0657 complexed with pseudo-ligand molecules will be described elsewhere. We anticipate that our structural study of BC0657 will make a significant contribution to the elucidation of ligand-induced transcriptional regulatory mechanisms of MepR.

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