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Overexpression, crystallization and preliminary X-ray crystallographic analysis of hypothetical protein SAV0479 from *Staphylococcus aureus* Mu50

SAV0479, a hypothetical protein from the Mu50 strain of methicillin- and vancomycin-resistant *Staphylococcus aureus*, was selected for structure and function determination as part of a structural genomics project. Here, the cloning, overexpression, purification and crystallization of SAV0479 are reported. Crystals were obtained by the hanging-drop vapour-diffusion method and diffraction data were collected to a resolution of 2.8 Å. The crystals belonged to space group $P3_121$, with unit-cell parameters $a = b = 81.48$, $c = 82.53$ Å. Three monomers of SAV0479 are present in each asymmetric unit.

1. Introduction

Staphylococcus aureus bacteria have developed extensive antibiotic resistance. An example of this phenomenon is the methicillin-resistant *S. aureus* (MRSA), which was isolated in 1961 (Jevons, 1961). *S. aureus* has become resistant to new antibiotics through mutations and by the acquisition of exogenous genes (Kuroda *et al.*, 2001; Lowy, 1998; Chambers, 1997; Brumfitt & Hamilton-Miller, 1989). To protect patients from the methicillin-resistant strain, glycopeptides, such as vancomycin, are being used as the therapeutic drug of choice for MRSA infections. As a result, vancomycin-resistant strains (VRSA) have emerged rapidly (Avison *et al.*, 2002; Hiramatsu, Hanaki *et al.*, 1997; Howe *et al.*, 1998; Ploy *et al.*, 1998; Tiwari & Sen, 2006). One vancomycin-resistant MRSA strain was reported in Japan in the early 1990s (Noble *et al.*, 1992). Since then many laboratories have reported vancomycin-resistant strains (Hiramatsu, Aritaka *et al.*, 1997; Palazzo *et al.*, 2005; Goodman *et al.*, 1997).

Several studies have been done to understand the mechanism of vancomycin resistance in methicillin-resistant *S. aureus* (Hanaki *et al.*, 1998). Many genes and protein database entries of different strains of *S. aureus* are available (GenBank accession numbers BA000017, BA000018 and BA000033 for *S. aureus* Mu50, *S. aureus* N315 and *S. aureus* MW2, respectively) (Kuroda *et al.*, 2001; Gill *et al.*, 2005). Even though genome sequencing sheds some light on many proteins, a considerable number of protein sequences remain designated as 'hypothetical proteins' with very few or no functional studies associated with them.

Using the GenBank and Comprehensive Microbial Resource, CMR (<http://cmr.jcvi.org>), we chose hypothetical proteins which might provide new information about antibiotic-resistant pathogens and possible targets for drug development. In the search for functional proteins from the methicillin- and vancomycin-resistant strain *S. aureus* Mu50, we selected some of the genes corresponding to hypothetical proteins in the MRSA strain. The *S. aureus* Mu50 strain is resistant to methicillin and vancomycin and almost all antibiotics including even the potent β -lactams (Jevons, 1961; Kuroda *et al.*, 2001). The present study focuses on one of these hypothetical proteins, SAV0479 from *S. aureus* Mu50 strain.

2. Material and methods

2.1. Cloning, expression and purification of SAV0479

The gene for SAV0479 was amplified using PCR from the whole genomic DNA of *S. aureus* Mu50. The amplified DNA fragment was

Table 1

Data-collection statistics.

Values in parentheses are for the highest-resolution shell.

X-ray source	Beamline NW12A, Photon Factory, Japan
Space group	$P3_121$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = b = 81.48$, $c = 82.53$, $\alpha = \beta = 90.0$, $\gamma = 120.0$
Wavelength ($\text{\AA}$)	1.0000
Oscillation per image ($^\circ$)	1
Total rotation range ($^\circ$)	270
Temperature (K)	100
Resolution range ($\text{\AA}$)	30–2.8
$R_{\text{merge}}^\dagger$ (%)	7.1 (41.4)
No. of observed reflections ($>1\sigma$)	123166
No. of unique reflections	8154
Completeness (%)	99.9 (99.6)
$\langle I/\sigma(I) \rangle$	41.95 (3.93)
Multiplicity ‡	15.1 (9.9)
Mosaicity	1.15
Monomers per asymmetric unit	3

$^\dagger R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I(hkl)$ is the intensity of reflection hkl , $\sum_{hkl}$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection hkl . $^\ddagger N_{\text{obs}}/N_{\text{unique}}$.

digested using *NdeI/XhoI* restriction sites and ligated with the pET-21a(+) vector (Novagen, Germany) using T4 DNA ligase (Takara Inc.). The presence of the insert was confirmed by DNA sequencing. The resulting construct contained one amino acid (methionine) at the N-terminal as a result of the *NdeI* site and three amino acids (arginine, leucine and glutamate) at the C-terminal site as a result of the *XhoI* site along with a hexahistidine tag to facilitate purification. Therefore, the overexpressed protein contained the following sequence, beginning from the N-terminus: (*NdeI* site, M)–(SAV0479, residues 1–105)–(*XhoI* site, RLE)–(hexahistidine tag). Overall the entire sequence contains 115 amino acids. The recombinant vector was then overexpressed in *Escherichia coli* BL21(DE3) cells. The cells were grown at 310 K until the OD_{600} reached 0.5. Protein overexpression was induced by adding isopropyl β -D-1-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM, after which the cells were incubated for an additional 4 h (SI-900R shaking incubator, Jeio Tech Inc., Korea). The cells were harvested by centrifugation at 4293g for 10 min.

Figure 1

SDS-PAGE showing purification on a Ni^{2+} affinity column. Lane 1, supernatant of sonicated SAV0479 just before loading onto the Ni^{2+} affinity column; lane 2, flow-through of the sample, showing complete binding of SAV0479 onto the column; lane 3, flow-through from the washing buffer containing 60 mM imidazole; lanes 4–7, elution profile of SAV0479 from FPLC Ni^{2+} -affinity column by imidazole gradient. Fractions 5 to 7 were used for protein crystallization trials. Lane M, molecular-mass marker.

The harvested cells were resuspended in lysis buffer (20 mM Tris–HCl, 500 mM NaCl pH 7.9) and lysed at 277 K using an ultrasonic processor (Cole Parmer Inc.) for 30 min with a 3 s ON/OFF cycle. The cell lysate was centrifuged at 6708g for 1 h. The supernatant was passed through a 0.45 μm pore-size membrane (Millex-HV filter unit, Millipore, USA) to remove any insoluble particles. The flow-through was loaded onto an open Ni^{2+} –NTA column (Qiagen, USA; 3 ml of resin per litre of cell culture) pre-equilibrated with lysis buffer. The column was washed with a 5 $\times$ excess volume wash buffer (20 mM Tris–HCl, 500 mM NaCl, 60 mM imidazole pH 7.9). The protein of interest, SAV0479, was eluted with a 0–0.5 M imidazole gradient (Fig. 1). The fractions containing SAV0479 were dialysed using a 3000 molecular weight cutoff (MWCO) cellulose membrane against the final buffer condition of 20 mM HEPES, 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM β -mercaptoethanol, 0.1 mM phenylmethanesulfonyl fluoride pH 7.0. The purity of the protein was judged to be of more than 95% as shown by SDS-PAGE analysis.

The concentration of protein was determined by the Bradford assay and the protein concentration of 10 mg ml $^{-1}$ was used for crystal screening (Bradford, 1976).

2.2. Crystallization and X-ray data collection

Crystallization conditions were obtained initially using commercially available crystallization screening kits from Hampton Research (USA) and Emerald BioSystems (USA). Crystallization conditions were screened by the hanging-drop vapour-diffusion method. 1 μl protein solution was mixed with an equal volume of buffer on a siliconized cover slip and the mixture was then equilibrated over 500 μl of reservoir solution at 293 K. The crystallization plates were observed after 1 d. Crystals grown in a solution consisting of 2.0 M NaCl, 0.1 M sodium acetate pH 4.6 were reproducible. Single crystals were obtained by varying the NaCl concentration and pH over a wide range. The crystals were then screened against a set of additives (Additive Screen Kit, Hampton Research, USA). The final optimized crystallization conditions were 1.8 M NaCl, 0.1 M sodium acetate pH 4.2, 1 mM taurine. The needle-shaped crystals grew to largest dimensions of 1.0 $\times$ 0.2 $\times$ 0.1 mm (Fig. 2).

For the cryogenic experiments, 4.5 M NaCl with 0.1 M sodium acetate pH 4.2 was used as a suitable cryoprotectant. The crystals

Figure 2

Single crystal of SAV0479 grown over 1–2 d in 1.8 M NaCl, 0.1 M sodium acetate pH 4.2, 1 mM taurine. The crystal dimensions are approximately 1.0 $\times$ 0.2 $\times$ 0.1 mm.

Figure 3

X-ray diffraction image from a SAV0479 crystal. Resolution rings from the centre outwards correspond to 40.00, 20.00, 8.00, 4.00 and 2.80 Å, respectively.

were soaked in this cryoprotectant solution for 10 s and flash-cooled in a liquid-nitrogen stream before data collection.

X-ray diffraction data were collected to a resolution of 2.8 Å at 100 K using an ADSC Quantum210 CCD detector, scanning a total of 360° rotations in ω on synchrotron beamline NW12A at the Photon Factory, Japan. The X-ray beam had a single wavelength of 1.0 Å (Fig. 3). 360 images were collected for the full data set. Each image was recorded with an exposure of 1.5 s per 1° oscillation range. The data set was indexed and integrated, and images from 31 to 300 were used for scaling with the *HKL-2000* program suite (Otwinowski & Minor, 1997). Molecular replacement was performed using *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* program suite (Winn *et al.*, 2011). Data-collection statistics are given in Table 1.

3. Results and discussion

The recombinant SAV0479 from *S. aureus* (MRSA Mu50 strain) was overexpressed in *E. coli* in soluble form with an approximate yield of 10 mg l⁻¹ LB medium. The molecular weight of the protein was estimated to be 11.5 kDa by *ProtParam* (<http://www.expasy.org/tools/protparam.html>, Gasteiger *et al.*, 2003). The protein gene was overexpressed in a pET-21a(+) vector with a hexahistidine tag at the C-terminus. As a result, ten extra amino acids were inserted in the native protein. The resulting sequence of SAV0479 is as follows: MMRGGGNMQMMKQMOKKMAQEKEKLKEERIVGT-AGGGMVAVTVTGHEKVEVDVEIKEEAVDPDDIEMQLDLVLA-ATNEAMNKADELTQERLGKHTQGLNIPGMPLHHHHHH. The presence of the C-terminal tag did not prevent the protein from forming crystals. The best crystals were grown in 1.8 M NaCl, 0.1 M sodium acetate pH 4.2, 1 mM taurine over 1–2 d. X-ray diffraction data were collected to 2.8 Å resolution. The data-collection statistics are summarized in Table 1.

A total of 123 166 measured reflections were merged into 8154 unique reflections, resulting in an R_{merge} of 7.1%. The crystals belong

to the $P3_121$ space group, with unit-cell parameters of $a = b = 81.48$, $c = 82.53$ Å and $\alpha = \beta = 90.0^\circ$, $\gamma = 120.0^\circ$. Three monomers of the protein are present in each asymmetric unit, with a calculated crystal volume per protein weight (V_M) of $2.04 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 39.89% (Matthews, 1968).

The structure of SAV0479 from *S. aureus* (MRSA Mu50 strain) was determined by the molecular-replacement method using the *CCP4* program suite with one monomer (chain A) of Cth-383 from *Clostridium thermocellum* (PDB entry 1ybx, Southeast Collaboratory for Structural Genomics, unpublished work) as the search model. The String database for protein–protein interactions (<http://string-db.org>) and PDBsum database (<http://www.ebi.ac.uk/pdbsum>) had suggested PDB entry 1ybx as a homology model for SAV0479 because of a 51.6% sequence identity. Model building and further refinement are in progress.

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