Expression, purification, crystallization and X-ray crystallographic analysis of the periplasmic binding protein VatD from *Vibrio vulnificus* M2799

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Vibrio vulnificus is a halophilic marine microorganism which causes gastroenteritis and primary septicaemia in humans. An important factor that determines the survival of *V. vulnificus* in the human body is its ability to acquire iron. VatD is a periplasmic siderophore-binding protein from *V. vulnificus* M2799. The current study reports the expression, purification and crystallization of VatD. Crystals of both apo VatD and a VatD–desferrioxamine B–Fe³⁺ (VatD–FOB) complex were obtained. The crystal of apo VatD belonged to space group *P*6₄22, while the crystal of the VatD–FOB complex belonged to space group *P*2₁. The difference in the two crystal forms could be caused by the binding of FOB to VatD.

1. Introduction

Vibrio vulnificus is a halophilic marine microorganism which causes gastroenteritis and primary septicaemia in humans. The septicaemia is often acquired by eating raw oysters or shellfish, and wound infections are associated with the exposure of wounds to seawater (Blake *et al.*, 1979; Tacket *et al.*, 1984; Klontz *et al.*, 1988). Primary septicaemia is often associated with patients who have diseases predisposing them to iron overload, such as liver cirrhosis, haemochromatosis and alcoholism, or who are immunocompromised (Johnston *et al.*, 1985). *V. vulnificus* sequesters iron through the biosynthesis and secretion of a low-molecular-weight chelating compound called a siderophore (Morris *et al.*, 1987; Litwin *et al.*, 1996). *V. vulnificus* M2799 produces a catecholate siderophore called vulnibactin (Okujo *et al.*, 1994). The vulnibactin-mediated iron-uptake system plays an important role in the growth of *V. vulnificus* M2799 under low iron-concentration conditions (Kawano *et al.*, 2013). Furthermore, the vulnibactin export system is composed of VV1_0612 TolC and several resistance nodulation-division proteins, including the VV1_1681 protein (Kawano *et al.*, 2014). Vulnibactin chelates ferric iron in the environment, and vulnibactin–Fe³⁺ is imported to the periplasm through the specific outer membrane receptor VuuA (Webster & Litwin, 2000). Subsequently, vulnibactin–Fe³⁺ is captured by a periplasmic binding protein (PBP), FatB, and is transported through the inner membrane by an ABC transporter. In a previous study, we clarified that the VatD protein, which functions as a periplasmic ferric aerobactin-binding protein, participates in the ferric vulnibactin-uptake system in the absence of FatB (Kawano *et al.*, 2013). VatD can capture both ferric aerobactin and ferric vulnibactin. Therefore, the

Table 1
Macromolecule-production information.

Source organism	<i>V. vulnificus</i> M2799
DNA source	Genomic DNA
Forward primer (vatD_Nc)	ATGTCCATGGACATCACCCACGAAATG
Reverse primer (vatD_Xh)	ATGACTCGAGTCATTGTGGCTGGGT
Expression vector	pProEX HTa
Expression host	<i>E. coli</i> BL21(DE3)pLysS
Complete amino-acid sequence of the construct produced	MSYYHHHHHDYDIPPTTENLYFQGAMDPDITHEM-GTTSFETTPKKVVALDWLTVLVLGIELEG-VANISGYQQWVAEPHLNADAIDVGSRRPNLE-LLSNIKPDVILISKHLAAAYEPLSKIAPVLVY-SVYSEDKPLESAKRITRSLGKLFQEQAEQ-VIAQTDQRLTANGAKITSAGKADKPLLFAEFI-NDKTLRIHSEGLAQDTINAMGLKNDWQEPN-LWGFTTTGTEKLAHQKANVMIFGPLSQEERQ-QLTQSPLWQAMEFSRSTDSVYELPAITWFGGLL-AAQRLSDHITGRLTQFPQ

structural analysis of VatD will be important for elucidation of the iron-uptake system in *V. vulnificus* M2799. Here, we report the expression, purification, crystallization and preliminary X-ray crystallographic analysis of *V. vulnificus* VatD. Furthermore, the complex of VatD with desferrioxamine B-Fe³⁺ (FOB) was also analysed.

2. Materials and methods

2.1. Macromolecule production

The expression plasmid pProVatD coding for the mature VatD protein was constructed as follows. Two oligonucleotide primers (vatD_Nc and vatD_Xh) were synthesized which were modified to contain NcoI and XhoI recognition sites to facilitate in-frame cloning into the His-tagged protein expression vector pProEX HTa (Invitrogen). PCR was performed by KOD -Plus- DNA polymerase (Toyobo) with the genome of *V. vulnificus* M2799 as a template for 30 cycles consisting of 367 K for 15 s, 333 K for 30 s and 341 K for 1 min. The amplified DNA was digested by NcoI and XhoI, and the resulting fragment (857 bp) was inserted into the corresponding sites of pProEX HTa. The nucleotide sequence of the PCR fragment was confirmed by DNA sequencing. *Escherichia coli* BL21(DE3)pLysS cells harbouring pProVatD were induced with 1.0 mM isopropyl β -D-1-thiogalactopyranoside at an OD₆₀₀ of 0.7 and further incubated overnight at 293 K. The cells were disrupted by sonication and the lysate was centrifuged at 30 000 rev min⁻¹ for 30 min at 277 K. The supernatant was loaded onto an Ni Sepharose 6 Fast Flow column (GE Healthcare) equilibrated with 20 mM Tris-HCl pH 7.5 containing 300 mM NaCl and 50 mM imidazole. The column was washed with five bed volumes of the same buffer. The His-tagged VatD (HisVatD) protein was eluted with 20 mM Tris-HCl pH 7.5 containing 300 mM NaCl and 300 mM imidazole, and treated with AcTEV protease (Invitrogen) overnight at 277 K to obtain VatD. To remove the His tag, the sample was reloaded onto an Ni Sepharose column and the non-adsorbed fraction was pooled. The fraction was dialyzed against 20 mM Tris-HCl pH 8.0 and applied onto a RESOURCE Q column (GE Healthcare). The column was

eluted with a linear gradient of 0–1.0 M NaCl in 20 mM Tris-HCl pH 8.0. The VatD protein was collected and further purified by HiLoad 16/600 Superdex 75 pg (GE Healthcare) column chromatography. The N-terminal amino-acid sequence of VatD was confirmed by protein sequencing. The purified apo VatD protein was concentrated to 9 mg ml⁻¹ in 20 mM Tris-HCl pH 7.5 containing 150 mM NaCl using an Amicon Ultra-15 centrifugal filter device (molecular-weight cutoff 10 kDa). Macromolecule-production information is summarized in Table 1.

2.2. Crystallization

Initial crystallization trials were carried out with the commercially available sparse-matrix screening kits Index (Hampton Research), Wizard 1, Wizard 2 (Emerald Bio), MCSG-1 and MCSG-2 (Microlytic) using the sitting-drop vapour-diffusion method at 293 K. The protein solution (0.5 μ l) was mixed with an equal volume of reservoir solution and then equilibrated against 60 μ l reservoir solution. Crystals of apo VatD appeared under condition No. 4 from MCSG-1 (the same condition as No. 17 in Wizard 2), condition No. 56 from MCSG-1 and condition No. 32 from MCSG-2. The best crystals were obtained using a reservoir consisting of 0.1 M Tris-HCl pH 7.0, 0.2 M MgCl₂, 2.5 M NaCl.

For the crystallization of VatD-FOB, apo VatD was mixed with the FOB complex, which was prepared from iron(III) chloride and desferrioxamine B mesylate (Sigma-Aldrich), in a 1:3 molar ratio overnight at 277 K. Formation of the VatD-FOB complex was verified by the spectral changes in the tryptophanyl fluorescence. Crystallization screening of VatD-FOB was carried out in the same way as for apo VatD. Crystals of VatD-FOB appeared using a reservoir consisting of 0.1 M bis-tris pH 6.5, 50 mM MgCl₂, 30% (w/v) polyethylene glycol (PEG) 3350. Crystallization information is summarized in Table 2.

Figure 1
SDS-PAGE of purified VatD. Lane 1 contains molecular-weight marker (labelled in kDa). Lane 2 contains purified apo VatD (31 kDa).

Table 2
Crystallization.

	Apo VatD	VatD-FOB
Method	Sitting-drop vapour diffusion	Sitting-drop vapour diffusion
Plate type	96-well	96-well
Temperature (K)	293	293
Protein concentration (mg ml ⁻¹)	9	9
Buffer composition of protein solution	20 mM Tris pH 7.5, 150 mM NaCl	20 mM Tris pH 7.5, 150 mM NaCl
Composition of reservoir solution	0.1 M Tris pH 7.0, 0.2 M MgCl ₂ , 2.5 M NaCl	0.1 M bis-tris pH 6.5, 50 mM MgCl ₂ , 30%(w/v) PEG 3350
Volume and ratio of drop	1.0 µl (1:1)	1.0 µl (1:1)
Volume of reservoir (µl)	60	60

2.3. Data collection and processing

Crystals of apo VatD and VatD-FOB were soaked in the crystallization solution supplemented with 20%(v/v) glycerol and were mounted in a cryoloop and flash-cooled in a stream of gaseous nitrogen at 100 K. X-ray diffraction data were collected from apo VatD crystals on beamline BL38B1 at SPring-8, Japan, which was equipped with an ADSC Quantum 315r CCD detector. Data collection was performed at a wavelength of 1.0 Å with a total oscillation range of 180°. Each diffraction image was obtained with an oscillation angle of 1.0°

(a)

(b)

Figure 2
(a) Crystals of apo VatD. The dimensions of the crystals are 0.2 × 0.2 × 0.1 mm. (b) Crystals of VatD-FOB. The dimensions of the crystals are 0.4 × 0.2 × 0.1 mm.

Table 3
Data collection and processing.

Values in parentheses are for the outer shell.

	Apo VatD	VatD-FOB
Diffraction source	BL38B1, SPring-8	Rigaku FR-E ⁺
Wavelength (Å)	1.0000	1.54
Temperature (K)	100	100
Detector	ADSC Quantum 315r CCD	R-Axis VII
Crystal-to-detector distance (mm)	320	115
Rotation range per image (°)	1	0.5
Total rotation range (°)	180	180
Exposure time per image (s)	30	360
Space group	<i>P</i> 6 ₂ 22	<i>P</i> 2 ₁
<i>a</i> , <i>b</i> , <i>c</i> (Å)	151.76, 151.76, 76.14	34.80, 57.90, 62.70
α , β , γ (°)	90, 90, 120	90, 95, 90
Mosaicity (°)	0.3	0.3
Resolution range (Å)	50.0–2.60 (2.69–2.60)	26.3–1.85 (1.92–1.85)
Total No. of reflections	98928	72517
No. of unique reflections	59929	20814
Completeness (%)	100 (100)	97.6 (95.4)
Multiplicity	5.8 (5.7)	3.48 (3.32)
$\langle I/\sigma(I) \rangle$	13.6 (3.7)	10.6 (2.4)
$R_{\text{merge}}^{\dagger}$	0.061 (0.379)	0.085 (0.413)
Overall <i>B</i> factor from Wilson plot (Å ²)	37.9	22.2

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the *i*th measurement of reflection *hkl* and $\langle I(hkl) \rangle$ is the weighted mean of all measurements of reflection *hkl*.

and an exposure time of 30 s. The diffraction data for apo VatD were processed and scaled with the *HKL-2000* program package (Otwinowski & Minor, 1997). Data for VatD-FOB were collected on a Rigaku FR-E⁺ rotating copper-anode X-ray generator equipped with an Osmic confocal mirror and an R-Axis VII image-plate scanner. The diffraction data for VatD-FOB were processed and scaled with *CrystalClear* (Rigaku). Details of the data collection and processing and statistics describing the quality of the data are given in Table 3.

3. Results and discussion

Recombinant VatD was purified to homogeneity using Ni-affinity, anion-exchange and gel-filtration chromatography. The purity of the protein was checked by SDS-PAGE with Coomassie staining (Fig. 1). The molecular mass of VatD calculated from the deduced amino-acid sequence is in reasonable agreement with that estimated by SDS-PAGE (31 kDa). The protein solution was concentrated to a sufficient level for crystallization. Crystals of apo VatD and VatD-FOB

were obtained under several conditions from commercial screening kits. After a series of optimization experiments, the quality of the crystals was improved to a suitable level for X-ray analysis (Fig. 2).

X-ray diffraction data for apo VatD and VatD-FOB were collected to resolutions of 2.60 and 1.85 Å, respectively, from flash-cooled crystals (Fig. 3). The crystals of apo VatD belonged to space group $P6_422$, with unit-cell parameters

$a = b = 151.8$, $c = 76.1$ Å. According to the unit-cell parameters and the molecular weight of VatD, solvent-content analysis suggested one molecule per asymmetric unit, with a V_M value of $4.03 \text{ Å}^3 \text{ Da}^{-1}$ and a solvent content of 69.5% (Matthews, 1968). The crystals of VatD-FOB belonged to space group $P2_1$, with unit-cell parameters $a = 34.8$, $b = 57.9$, $c = 62.7$ Å, $\alpha = 90.0$, $\beta = 95.0$, $\gamma = 90.0^\circ$. The V_M value of $2.03 \text{ Å}^3 \text{ Da}^{-1}$ indicated that the VatD-FOB crystal contained one protein molecule per asymmetric unit. The data-collection statistics are shown in Table 3. Interestingly, the crystallization condition and crystal form of VatD-FOB were different from those of apo VatD. These differences could be caused by a conformational change of VatD owing to the binding of FOB.

The molecular-replacement procedure was applied to the structure determination of apo VatD and VatD-FOB and was performed using FhuD from *E. coli* (PDB entry 1efd; Clarke *et al.*, 2000) as a search model with *MOLREP* (Vagin & Teplyakov, 2010) from the *CCP4* suite (Winn *et al.*, 2011). The initial maps of both models showed clear electron density. The structures of both apo VatD and VatD-FOB are under construction.

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Figure 3
(a) A diffraction image from an apo VatD crystal obtained on BL38B1 at SPring-8. (b) A diffraction image from a VatD-FOB crystal obtained using an in-house X-ray source (Rigaku FR-E⁺, Cu $K\alpha$ radiation).

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