YopT domain of the PfhB2 toxin from *Pasteurella multocida*: protein expression, characterization, crystallization and crystallographic analysis

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Pasteurella multocida causes respiratory-tract infections in a broad range of animals, as well as opportunistic infections in humans. *P. multocida* secretes a multidomain toxin called PfhB2, which contains a YopT-like cysteine protease domain at its C-terminus. The YopT domain of PfhB2 contains a well conserved Cys–His–Asp catalytic triad that defines YopT family members, and shares high sequence similarity with the prototype YopT from *Yersinia* sp. To date, only one crystal structure of a YopT family member has been reported; however, additional structural information is needed to help characterize the varied substrate specificity and enzymatic action of this large protease family. Here, a catalytically inactive C3733S mutant of PfhB2 YopT that provides enhanced protein stability was used with the aim of gaining structural insight into the diversity within the YopT protein family. To this end, the C3733S mutant of PfhB2 YopT has been successfully cloned, overexpressed, purified and crystallized. Diffraction data sets were collected from native crystals to 3.5 Å resolution and a single-wavelength anomalous data set was collected from an iodide-derivative crystal to 3.2 Å resolution. Data pertaining to crystals belonging to space group $P3_1$, with unit-cell parameters $a = 136.9$, $b = 136.9$, $c = 74.7$ Å for the native crystals and $a = 139.2$, $b = 139.2$, $c = 74.7$ Å for the iodide-derivative crystals, are discussed.

1. Introduction

Pasteurella multocida is a Gram-negative respiratory pathogen (Harper *et al.*, 2003). It encodes several secreted virulence factors, including a relatively uncharacterized putative filamentous hemagglutinin, PfhB2 (Harper *et al.*, 2003, 2006; Bojesen *et al.*, 2004; Tatum *et al.*, 2005; Pruimboom *et al.*, 1996; Blöcker *et al.*, 2006; Siegert *et al.*, 2013; Johnson *et al.*, 2013). PfhB2 is expressed as a 3919-amino-acid single polypeptide chain that undergoes processing during secretion from the bacterium. It is a close homologue of the IbpA toxin secreted by the respiratory pathogen *Histophilus somni*, which causes host-cell cytotoxicity and is required for breaching the alveolar barriers of the host (Mattoo *et al.*, 2011; Worby *et al.*, 2009; Zekarias *et al.*, 2010). PfhB2 shares an identical domain structure with *H. somni* IbpA. Specifically, following an N-terminal secretion signal, PfhB2 consists of an FHA (filamentous haemagglutinin)-like domain, followed by two consecutive Fic (filamentation induced by cAMP) domains (Fic1 and Fic2) and a YopT (*Yersinia* outer protein T)-like domain. The FHA-like domain of PfhB2 is predicted to play a role in attachment, akin to *Bordetella pertussis* FHA (Scheller & Cotter, 2015). Additionally, we have previously demonstrated that the Fic domains of PfhB2 are active adenyl-ly-

transferases that add an adenosine monophosphate (AMP) to the mammalian Rho GTPases RhoA, Rac1 and Cdc42, thereby inactivating them (Mattoo *et al.*, 2011). This Fic-mediated adenylation/AMPylation reaction causes cytoskeletal collapse and is predicted to be important for *P. multocida* to evade phagocytosis. However, little is known about the YopT-like domain of PfhB2.

PSI-BLAST analysis reveals that the YopT domain of PfhB2 (PfhB2-YopT) bears high sequence identity to the YopT domain of IbpA (81%) and to the prototype YopT from *Y. enterocolytica* (38%). *Y. enterocolytica* YopT is a cysteine protease that cleaves Rho GTPases, resulting in disruption of the actin cytoskeleton and causing cell rounding of host cells (Schmidt, 2011; Iriarte & Cornelis, 1998). The proteolytic activity of YopT depends on a conserved Cys–His–Asp catalytic triad that is characteristic of various cysteine proteases (Zhu *et al.*, 2004; Shao *et al.*, 2003). Based on its secondary structure and the position of the invariant Cys/His/Asp residues, YopT and its homologs are classified as members of the CA clan of cysteine proteases (Schmidt, 2011; Shao *et al.*, 2003; Barrett & Rawlings, 2001). Accordingly, we predict that PfhB2 is a CA clan cysteine protease by virtue of its YopT-like domain.

The crystal structure of only one YopT family cysteine protease, that of AvrPphB from *Pseudomonas syringae*, has thus far been determined (PDB entry 1ukf; Zhu *et al.*, 2004). Like YopT, AvrPphB also possesses a Cys–His–Asp catalytic triad; however, unlike YopT, AvrPphB does not cleave Rho GTPases. Instead, it functions as an autoproteolytic enzyme that enables it to induce a hypersensitivity response in plants (Shao *et al.*, 2002). Computational structure predictions show that the catalytic triad of YopT is structurally similar to the catalytic core of AvrPphB (Hasan *et al.*, 2014); however, information regarding substrate specificity cannot be gleaned from such analyses. Therefore, the structural determination of additional YopT family members is likely to offer critical insights into how this large protein family catalyzes the cleavage of diverse protein targets.

The secondary structure of PfhB2-YopT is predicted to resemble those of AvrPphB and YopT, with a conserved catalytic triad. Thus, we predict that PfhB2-YopT is an enzymatically active cysteine protease. However, PfhB2-YopT does not appear to target host Rho GTPases (our unpublished observations). We therefore sought to determine the structure of PfhB2-YopT in order to gain insight into its substrate recognition and catalytic activity. We cloned, overexpressed and purified wild-type PfhB2-YopT (PfhB2-YopT_{WT}) and its predicted catalytically inactive C3733S mutant (PfhB2-YopT_{C3733S}). Crystallization screening of PfhB2-YopT_{WT} failed to generate crystals. In contrast, PfhB2-YopT_{C3733S} crystallized under several conditions. A scan of structures deposited in the Protein Data Bank (PDB) failed to identify any homologs of the PfhB2-YopT structure, suggesting that the structure of PfhB2-YopT is likely to be novel. Here, we report the collected native data set for the obtained PfhB2-YopT_{C3733S} crystals, as well as an iodide-derivative data set, to allow phasing and structure determination.

Table 1

Macromolecule-production information.

Source organism	<i>P. multocida</i> Pm70
DNA source	Genomic DNA
Forward primer	5'-GCTGGAGGATCCATGGCTTCAGTAGCA GAATATG-3'
Reverse primer	5'-CGACGCTCTCGAGTTACTTGGTTTCTGC GTG-3'
Cloning vector	pSMT3
Expression vector	pSMT3
Expression host	<i>E. coli</i> BL21 (DE3)
Complete amino-acid sequence of the construct produced	SVAEYGGVEVSFKYAQSKGEVYKEIVKHVDI QHGVSESTCAHWIANKVSSQGEDFWNTM YEGGKKGHLKQEAIDSIKKLQTEFMQSG SATQQFKLTDNWLQEQGVVPKEKKVGD SRDEVAGTVSKSDISALTKAILDTGSD TAGAKKISINLEGGSHTVSALVQGEKVV FFDPNFGEMTFPSHQKFESWLKEAFWEK SGYAGKKEGKRFFNVVNYHAETK

2. Materials and methods

2.1. Cloning and protein purification

The primers for cloning and site-directed mutagenesis are indicated in Table 1. Using *P. multocida* genomic DNA as a template, DNA corresponding to the YopT domain of PfhB2 was amplified and cloned into the BamHI and XhoI sites of pSMT3 for protein expression with an N-terminal His₆-SUMO tag (Xiao *et al.*, 2010). PfhB2-YopT_{C3733S} was generated using Q5 Site-Directed Mutagenesis (New England Biosciences). The constructs were confirmed by DNA sequencing and were transformed into *Escherichia coli* BL21 (DE3) cells for protein expression. Bacteria grown in 1 l LB supplemented with 50 µg ml⁻¹ kanamycin were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at an OD₆₀₀ of 0.8 for 3 h at 37°C. The cells were harvested by centrifugation at 5000g for 15 min at 4°C. Pellets resuspended in lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 5 mM imidazole, 1 mM phenylmethanesulfonylfluoride) were lysed by sonication and the supernatant was collected following centrifugation at 27 000g for 40 min at 4°C. The supernatant was applied onto a 2 ml cobalt resin (Thermo Scientific) column pre-equilibrated with lysis buffer. The column was then washed with 20 ml wash buffer (50 mM Tris pH 8.0, 100 mM NaCl, 5 mM β-mercaptoethanol) and 10 ml lysis buffer to remove nonspecific proteins. Proteins were eluted with 10 ml elution buffer (50 mM Tris pH 8.0, 150 mM NaCl, 5 mM β-mercaptoethanol, 200 mM imidazole). The His₆-SUMO tag was cleaved from the purified proteins by incubation with ULP protease overnight at 4°C. Cleaved proteins were purified by reverse Co²⁺-affinity purification, where the flowthrough fraction contained the desired YopT proteins. PfhB2-YopT_{WT} and PfhB2-YopT_{C3733S} were further purified by size-exclusion chromatography (SEC) using a Superdex 16/600 GL column (GE Healthcare). Elution peaks corresponding to the purified proteins were collected and assessed for purity using SDS-PAGE on a 12% polyacrylamide gel. Prior to crystallization, the proteins were further concentrated to 15 mg ml⁻¹ using a 10 kDa cutoff Centricon column. The protein concentration was determined by measuring the absorbance at 280 nm.

Table 2
Crystallization.

Method	Hanging-drop vapor diffusion
Plate type	24-well plates from Hampton Research
Temperature (K)	293
Protein concentration (mg ml ⁻¹)	15
Buffer composition of protein solution	50 mM Tris pH 8.0, 150 mM NaCl, 5 mM β -mercaptoethanol
Composition of reservoir solution	0.2 M sodium acetate, 20% PEG 3350
Volume and ratio of drop	3 μ l (1:1)
Volume of reservoir (μ l)	500

2.2. Crystallization

Initial crystallization trials were conducted manually by the sitting-drop vapor-diffusion method in 96-well plates using screens from Hampton Research (Aliso Viejo, California, USA). Crystallization drops consisting of 1 μ l protein solution mixed with 1 μ l precipitant solution were equilibrated against 100 μ l reservoir solution at 293 K. Crystals of PfhB2-YopT_{C3733S} appeared within 24 h of incubation in various conditions: PEG/Ion screen condition Nos. 1 (0.2 M sodium fluoride, 20% PEG 3350), 2 (0.2 M potassium fluoride, 20% PEG 3350), 20 (0.2 M magnesium formate, 20% PEG 3350), 25 (0.2 M magnesium acetate, 20% PEG 3350) and 27 (0.2 M sodium acetate, 20% PEG 3350) and SaltRx screen conditions Nos. 75 (1.0 M magnesium sulfate hydrate, 0.1 M Tris pH 8.5) and 87 (0.6 M potassium sodium tartrate tetrahydrate, 0.1 M Tris pH 8.5). Crystals were further optimized in 24-well plates by varying the PEG and salt concentrations. Diffraction-quality crystals were obtained in 0.2 M magnesium acetate, 22% PEG 3350, 0.2 M sodium acetate, 22% PEG 3350. A crystallization summary is provided in Table 2.

Table 3
Data collection and processing.

Data set	Native	Iodide derivative
Diffraction source	23-ID-D, APS	23-ID-B, APS
Wavelength ($\text{\AA}$)	1.03	1.85
Temperature (K)	100	100
Detector	PILATUS3 6M	EIGER 16M
Crystal-to-detector distance (mm)	600	200
Rotation range per image ($^{\circ}$)	1.0	0.5
Total rotation range ($^{\circ}$)	100	358
Exposure time per image (s)	5	0.5
Space group	$P3_1$	$P3_1$
a, b, c ($\text{\AA}$)	136.9, 136.9, 74.7	139.2, 139.2, 74.7
α, β, γ ($^{\circ}$)	90, 90, 120	90, 90, 120
Matthews volume V_M ($\text{\AA}^3 \text{Da}^{-1}$)	2.44	2.49
Molecules in asymmetric unit	6	6
R_{meas}	0.134 (0.614)	0.124 (1.095)
Resolution range ($\text{\AA}$)	50–3.5 (3.63–3.55)	60.2–3.2 (3.32–3.26)
Total No. of reflections	66336 (3080)	85099 (3908)
No. of unique reflections	23480 (1141)	24950 (1261)
Completeness (%)	99.7 (99.7)	98.7 (98.3)
Multiplicity	2.8 (2.7)	3.4 (3.1)
$\langle I/\sigma(I) \rangle$	7.8 (1.5)	8.5 (1.4)
$CC_{1/2}$	0.955 (0.680)	0.994 (0.622)
$R_{\text{p.i.m.}}$	0.077 (0.358)	0.085 (0.763)
Overall B factor from Wilson plot ($\text{\AA}^2$)	93.9	66.7

2.3. Data collection and processing

To confirm that the crystals obtained were indeed of PfhB2-YopT_{C3733S}, we picked up a crystal from the crystallization drop, washed it in mother liquor and dissolved it in phosphate-buffered saline. The dissolved protein crystal was then analyzed by mass spectrometry. Specifically, the

Figure 1
Purification of *P. multocida* PfhB2-YopT. (a) SDS-PAGE analysis of PfhB2-YopT_{WT} purified after size-exclusion chromatography (SEC). Lane M, molecular-mass marker (labelled in kDa); lanes 1 and 2, SEC peak fractions. (b) SEC analysis of PfhB2-YopT_{WT} using a Superdex 16/600 GL column. (c) SDS-PAGE analysis of PfhB2-YopT_{C3733S} purified after SEC. Lane M, molecular-mass marker (labelled in kDa); lanes 1 and 2, SEC peaks corresponding to the 78 ml fraction; lane 3, the 87 ml SEC peak fraction. (d) SEC analysis of PfhB2-YopT_{C3733S} using a Superdex 16/600 GL column.

trypsin-digested protein sample was analyzed using an 1100 Series nanoLC system (Agilent Technologies, Santa Clara, California, USA) coupled to an LTQ Orbitrap XL mass spectrometer (Thermo Scientific, Waltham, Massachusetts, USA). Peptides were loaded onto a 0.3×5 mm 300SB-C18 enrichment column (Agilent) and washed for 5 min using 5% acetonitrile/0.1% formic acid in purified water before being switched inline with the analytical column. Peptides were separated using a $75 \mu\text{m} \times 150$ mm reversed-phase Zorbax 300SB-C18 (Agilent) column using a 65 min method at a flow rate of 300 nl min^{-1} . Mobile phase *A* was 0.1% formic acid in purified water, while mobile phase *B* consisted of 0.01% formic acid in acetonitrile. For the first 5 min, the column was equilibrated with 95% purified water/0.1% formic acid (mobile phase *A*) followed by a linear gradient of 5% *B* to 40% *B* in 40 min, 60% *B* in 43 min and 95% *B* in 46 min. The columns were held at 95% *B* for the next 4 min before being returned to 5% *B* for 15 min. The sample was injected into the mass spectrometer using a Nanospray Flex Series ion source

(Thermo Scientific) fitted with an emitter tip (New Objective). Data acquisition was performed in data-dependent positive mode monitoring the top eight precursors at 30 000 resolution. Precursors were fragmented by collision-induced dissociation (CID) using a normalized collision energy of 35%. The database-search analysis was performed using *Mascot Daemon* v2.5.1 (Matrix Science). The data were searched against a *P. multocida* database downloaded from UniProtKB.

Crystals were soaked in cryoprotectant consisting of 0.2 *M* sodium acetate, 20% PEG 3350, 15% PEG 400. Single crystals were picked up in cryoloops and flash-cooled in liquid nitrogen. Crystals were also flash-cooled directly from the drop without cryoprotectant. X-ray diffraction data were collected on GM/CA beamlines 23-ID-B and 23-ID-D at the Advanced Photon Source (APS), Argonne, Illinois, USA. The diffraction data for the native crystals were optimal in the absence of cryoprotectant. Diffraction data were processed and scaled using *HKL-2000* (Otwinowski & Minor, 1997), *XDS* (Kabsch, 2010) and the *xia2* automated data-processing

Figure 2
Crystal images of *P. multocida* PfhB2-YopT_{C3733S} obtained under various crystallization conditions. (a) 0.2 *M* sodium fluoride, 20% PEG 3350. (b) 0.6 *M* potassium sodium tartrate tetrahydrate, 0.1 *M* Tris pH 8.5. (c) 0.2 *M* magnesium acetate, 20% PEG 3350. (d) 0.2 *M* sodium acetate, 20% PEG 3350.

tool from the CCP4 package (Winn *et al.*, 2011; Evans, 2006; Leslie, 2006; Sauter *et al.*, 2004). Data statistics are presented in Table 3. As no homologous structures corresponding to the amino-acid sequence of PfhB2-YopT_{C3733S} had been deposited in the PDB, we also collected halide-derivative data from crystals soaked in iodide solution to overcome the phase problem. Crystals were quick-soaked for 30 s to 2 min in 250–500 mM potassium iodide dissolved in the cryoprotectant solution. Data were collected at a wavelength of 1.85 Å. The iodide-soaked crystals diffracted better than the native crystals, to 3.2 Å resolution. Data statistics are provided in Table 3. Data quality was assessed using *phenix.xtriage* in the PHENIX suite (Adams *et al.*, 2010).

3. Results and discussion

When assessed by SDS–PAGE analysis, bacterially expressed and purified PfhB2-YopT_{WT} and PfhB2-YopT_{C3733S} revealed a single Coomassie-stained protein band in each sample corresponding to the predicted molecular weight of 24.5 kDa (Figs. 1*a* and 1*c*). Following SEC purification, PfhB2-YopT_{WT} eluted as a single peak at an elution volume of 83 ml (Fig. 1*b*). However, crystallization attempts with PfhB-YopT_{WT} failed to yield any crystals. In contrast, PfhB2-YopT_{C3733S} eluted as two

peaks, with the bulk of the protein eluting at an elution volume of 78 ml and a smaller proportion eluting at 87 ml (Fig. 1*d*). Such an elution profile suggests that PfhB2-YopT_{C3733S} purifies as two conformationally distinct populations, with the peak at 78 ml possibly representing a dimer, thus stabilizing the protein and making it amenable to crystallization. Indeed, crystals of PfhB2-YopT_{C3733S} appeared within 24 h of incubation in various conditions: PEG/Ion screen condition Nos. 1 (0.2 M sodium fluoride, 20% PEG 3350; Fig. 2*a*), 2 (0.2 M potassium fluoride, 20% PEG 3350), 20 (0.2 M magnesium formate, 20% PEG 3350), 25 (0.2 M magnesium acetate, 20% PEG 3350) and 27 (0.2 M sodium acetate, 20% PEG 3350), and SaltRx screen condition Nos. 75 (1.0 M magnesium sulfate hydrate, 0.1 M Tris pH 8.5) and 87 (0.6 M potassium sodium tartrate tetrahydrate, 0.1 M Tris pH 8.5; Fig. 2*b*). Crystals were further optimized and diffraction-quality crystals were obtained in 0.2 M magnesium acetate, 22% PEG 3350 (Fig. 2*c*) and in 0.2 M sodium acetate, 22% PEG 3350 (Fig. 2*d*).

The identity of the protein crystals was confirmed using mass spectrometry, which indicated 4% coverage of the full-length PfhB2 protein (UniProt Q9CPH9_PASMU, PfhB2 from *P. multocida* strain Pm70), with all of the peptides corresponding to the C-terminal region containing the YopT

Figure 3
MS/MS analysis of PfhB2-YopT_{C3733S} crystals. Peak profiles of the peptides (a) LQTEFMQSGSATQQFK corresponding to amino acids 3776–3791 and (b) ISINLEGGSHTVSALVQGEK corresponding to amino acids 3847–3866 of *P. multocida* PfhB2 are shown, and confirm the identity of our protein crystals.

domain. The peak profiles of two representative peptides corresponding to the sequence of PfhB2-YopT are shown in Fig. 3. The crystals diffracted to 3.5 Å resolution and belonged to space group $P3_1$, with unit-cell parameters $a = 136.9$, $b = 136.9$, $c = 74.7$ Å (Fig. 4a).

We calculated the Matthews coefficient (V_M) as $2.44 \text{ Å}^3 \text{ Da}^{-1}$, allowing us to predict the presence of six molecules in the asymmetric unit, with a solvent content of 49.58% ($P = 42\%$; Matthews, 1968). Next, a self-rotation

function was calculated with *MOLREP* (Vagin & Teplyakov, 2010) using data between 46 and 4 Å resolution (Fig. 5). In addition to the single peak in the $\kappa = 120^\circ$ section owing to crystallographic symmetry, three noncrystallographic twofold axes parallel to the ab plane were present. Further, *phenix.xtriage* found a translation peak of $\sim 1/3$ the height of the origin peak in the self-Patterson function at $(u, v, w) \simeq (1/3, -1/3, 0)$. These results are consistent with an asymmetric unit composed of three dimers related by translations.

Figure 4 Diffraction images of PfhB2-YopT_{C373S} crystals. (a) Native crystals diffracting to 3.5 Å resolution. (b) Iodide-derivative crystals diffracting to 3.2 Å resolution.

Figure 5 Self-rotation function analysis. Stereographic projection of the self-rotation function calculated using data between 46 and 4 Å resolution at a radius of integration of 37 Å. Axes are indicated as follows: x is aligned with the crystal a axis and z is aligned with c^* .

Interestingly, we identified no homologous structures to that of PfhB2-YopTC_{3733S} in the Protein Data Bank (PDB) based on sequence alignment, and were therefore unable to use molecular replacement to solve our crystal structure. To circumvent this phase problem, we collected diffraction data from native crystals that had been quick-soaked with potassium iodide. Iodide soaking improved the diffraction quality, with the crystals diffracting to 3.2 Å resolution (Fig. 4b). Data processing indicated that the iodide-derivative crystals also belonged to space group *P*3₁, with unit-cell parameters *a* = 139.2, *b* = 139.2, *c* = 74.7 Å. While the iodide-derived crystals improved the resolution, they did not help in solving the phase problem using single anomalous dispersion (SAD). Repeating the crystallization screens for PfhB2-YopT_{WT} using the same conditions that had proved successful for crystallizing PfhB2-YopTC_{3733S} also failed to yield any results.

Studies are ongoing to obtain selenomethionine-derived protein crystals to allow phasing and structure solution. Given that YopT-like proteases are widespread in nature and are likely to act on diverse targets, our crystal structure determination of PfhB2-YopTC_{3733S} will provide critical insights into the structural diversity and substrate specificity of this enzyme family.

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