Rui Bao,^a Lothar Esser,^a
Annapurna Sadhukhan,^b
Manoj K. M. Nair,^b Dieter M.
Schifferli^b and Di Xia^{a*}

^aLaboratory of Cell Biology, Center for Cancer
Research, National Cancer Institute, NIH,
Bethesda, MD 20892-4256, USA, and
^bDepartment of Pathobiology, School of
Veterinary Medicine, University of
Pennsylvania, Philadelphia, PA 19104, USA

Correspondence e-mail: xiad@mail.nih.gov

Received 21 June 2012

Accepted 20 July 2012

Crystallization and preliminary X-ray diffraction analysis of PsaA, the adhesive pilin subunit that forms the pH 6 antigen on the surface of *Yersinia pestis*

Yersinia pestis has been responsible for a number of high-mortality epidemics throughout human history. Like all other bacterial infections, the pathogenesis of *Y. pestis* begins with the attachment of bacteria to the surface of host cells. At least five surface proteins from *Y. pestis* have been shown to interact with host cells. Psa, the pH 6 antigen, is one of them and is deployed on the surface of bacteria as thin flexible fibrils that are the result of the polymerization of a single PsaA pilin subunit. Here, the crystallization of recombinant donor-strand complemented PsaA by the hanging-drop vapor-diffusion method is reported. X-ray diffraction data sets were collected to 1.9 Å resolution from a native crystal and to 1.5 Å resolution from a bromide-derivatized crystal. These crystals displayed the symmetry of the orthorhombic space group $P222_1$, with unit-cell parameters $a = 26.3$, $b = 54.6$, $c = 102.1$ Å. Initial phases were derived from single isomorphous replacement with anomalous scattering experiments, resulting in an electron-density map that showed a single molecule in the crystallographic asymmetric unit. Sequence assignment was aided by residues binding to bromide ions of the heavy-atom derivative.

1. Introduction

The Gram-negative bacterium *Yersinia pestis* has caused bubonic and pneumonic plague responsible for three pandemics in human history and is still perceived as one of the most dangerous pathogens in the Enterobacteriaceae family (Parkhill *et al.*, 2001). As is the case for most pathogenic bacteria, the adherence of *Y. pestis* to host cells is critical for its infectivity and is established through specific binding of surface-exposed adhesive proteins such as fimbriae or fibrils to host tissues.

Psa, the pH 6 antigen, belongs to the $\gamma 3$ -fimbriae that assemble *via* the classic chaperone–usher pathway (Nuccio & Bäumlér, 2007) and is one of the major adhesive fibrils expressed on the surface of *Y. pestis* when the bacteria are grown at a temperature of 310 K and a pH of between 5 and 6.7 (Ben-Efraim *et al.*, 1961). During the process of fibril biogenesis, the adhesive PsaA subunit is translocated to the periplasm and forms a complex with the chaperone protein PsaB, which is then targeted to the membrane-bound usher protein PsaC for assembly. Mature Psa fibrils consist of many copies of PsaA that polymerize into flexible fibrillar organelles that are deployed on the bacterial surface (Lindler & Tall, 1993). The role of Psa in the pathogenesis of *Y. pestis* remains elusive; however, there is experimental evidence demonstrating its ability to mediate binding to the HEp-2 cell line derived from epidermoid carcinoma tissue and to three human respiratory-tract epithelial cell lines (Liu *et al.*, 2006; Yang *et al.*, 1996). Experiments have also suggested that Psa confers the bacteria with the ability to evade phagocytosis by host organisms during *Y. pestis* infection (Huang & Lindler, 2004).

Most interestingly, Psa fibrils are capable of binding to at least two cell-surface components: the phosphatidylcholine and galactosyl moieties of glycosphingolipids. These binding interactions can be effectively blocked by small molecules such as phosphocholine and galactose (Galván *et al.*, 2007; Payne *et al.*, 1998). By elucidating the

biochemical and structural basis of these multifaceted interactions, we hope to gain further insights into the mechanisms of *Y. pestis* pathogenesis. In particular, an understanding of the mechanism of Psa function may lead to the development of potential therapeutics that interfere with the infection process of *Y. pestis*. Here, we report the purification and crystallization of donor-strand complemented PsaA (dscPsaA) as well as the preliminary results of X-ray diffraction experiments on dscPsaA crystals. The structure of dscPsaA was solved by the single isomorphous replacement with anomalous scattering phasing method (SIRAS), as no suitable template was available for solution using the molecular-replacement approach.

2. Materials and methods

2.1. Expression and purification of recombinant donor-strand complemented PsaA

To recombinantly express dscPsaA (PsaA; GenBank accession code AAA27662.1), the expression vector pET22b::dsc18psaA(His)₆ was constructed. dscPsaA was produced by removing the first 30 residues that code for the periplasm-targeting signal peptide and by relocating residues 31–48, the donor strand, to the C-terminus following a DNKQ linker. A C-terminal LEHHHHHH tag was added for purification purposes. The resulting protein also contained an N-terminal artificial methionine. The plasmid pET22b::dsc18psaA(His)₆ was transformed into *Escherichia coli* strain BL21 (DE3) for expression. The bacterial culture was grown in Terrific Broth (Research Products International Corp., Mount Prospect, Illinois, USA) in the presence of 60 µg ml⁻¹ ampicillin (Sigma, St Louis, Missouri, USA) at 310 K until the OD₆₀₀ reached 0.8. The culture was cooled to 289 K for 1 h before protein expression was induced overnight with 1 mM IPTG (Sigma). The cell paste collected by centrifugation was suspended in a buffer consisting of 50 mM Tris-HCl pH 7.5, 150 mM NaCl, and the *E. coli* cells were disrupted by microfluidization (APV-2000; APV Americas, Lake Mills, Wisconsin, USA). The lysate was cleared by centrifugation at 26 000g for 30 min and the supernatant was filtered and loaded onto a 3 ml Ni-NTA Fast Flow column (Qiagen, Valencia, California, USA) pre-equilibrated with the same buffer. The Ni-NTA column was washed with three column volumes of the buffer supplemented with 50 mM imidazole. The target protein was eluted with the same buffer in the presence of 300 mM imidazole. The resulting protein was further purified by size-exclusion chromatography on a Superdex 75 column (GE Healthcare, Piscataway, New Jersey, USA) pre-equilibrated with the same buffer. Fractions containing dscPsaA were pooled and concentrated to a concentration of approximately 40 mg ml⁻¹ using a Centricon filter (10 kDa cutoff; Millipore, Billerica, Massachusetts, USA).

2.2. Crystallization and cryoprotection of crystals

Crystallization screens were carried out robotically using a Mosquito liquid dispenser (TTP LabTech, Cambridge, Massachusetts, USA) by the hanging-drop vapor-diffusion method at room temperature, mixing 125 nl protein solution with the same volume of reservoir buffer in a 96-well format and using commercial high-throughput screening kits (Hampton Research, Aliso Viejo, California, USA; Emerald BioSystems, Bainbridge Island, Washington, USA; Molecular Dimensions, Apopka, Florida, USA). Subsequent optimizations were performed manually by mixing 1 µl protein solution with 1 µl precipitant solution and the admixture was equilibrated against 500 µl reservoir solution using the hanging-drop vapor-diffusion method at 294 K. Optimization included varying the concentration of precipitants and the pH and the use of various types

of additive compounds and known ligands. Crystallization well solutions supplemented with different concentrations of glycerol, various PEGs, sucrose and salts were tested for cryoprotection of dscPsaA crystals by dipping the crystals directly into the solutions for various lengths of time followed by flash-cooling in liquid nitrogen.

2.3. Preparation of heavy-metal derivatives

A stabilization solution was devised for dscPsaA crystals consisting of 3 M sodium malonate pH 4.2. The halide-soak method for introducing heavy atoms into crystals was used by dipping dscPsaA crystals into the stabilization solution containing an additional 1.5 M NaBr for 30 s (Dauter & Dauter, 2007). Crystals were subsequently flash-cooled in liquid propane.

2.4. X-ray diffraction data collection and processing

X-ray diffraction images of dscPsaA crystals were collected on the SER-CAT BM beamline at the Advanced Photon Source (APS), Argonne National Laboratory (ANL) using a MAR CCD 225 detector. Data frames were indexed and diffraction spots were integrated and scaled using the *HKL-2000* program package (Otwinowski & Minor, 1997). Single isomorphous replacement with anomalous scattering (SIRAS) phasing was performed using the *PHENIX* program suite (Adams *et al.*, 2010).

3. Results and discussion

3.1. Protein purification, crystallization, cryoprotection and preliminary X-ray diffraction analysis

The dscPsaA protein was produced with its N-terminal 18-residue donor-strand removed, starting with residue Asn19 (the numbering is based on the mature form of the protein not including the signal peptide), and with a four-residue (DNKQ) linker followed by the 18-residue donor-strand and an LEHHHHHH tag added to its C-terminus. The dscPsaA protein thus constructed contains 145 residues with a molecular mass of 15.9 kDa. This protein was over-expressed in *E. coli* and purified to homogeneity from the cell extract by sequential nickel-affinity and size-exclusion chromatography. The protein was concentrated to 40 mg ml⁻¹ in 20 mM Tris-HCl pH 7.5,

Figure 1 SDS-PAGE, Western blot and blue native PAGE of purified dscPsaA. (a) SDS-PAGE analysis of purified dscPsaA. Molecular-mass markers are labeled in kDa. (b) Western blot with an antihistidine-tag antibody. (c) Blue native PAGE of purified dscPsaA at two concentrations.

100 mM NaCl. SDS-PAGE analysis of the concentrated dscPsaA sample showed additional higher molecular-weight smeared bands (Fig. 1*a*). Immunoblotting confirmed that the smeared upper band also originated from dscPsaA (Fig. 1*b*). Blue native PAGE of the sample at the lower concentrations of 5 and 10 mg ml⁻¹ showed two distinct bands (Fig. 1*c*), indicating that the sample was suitable for crystallization (Ma & Xia, 2008). Indeed, this dscPsaA preparation was able to produce crystals within 1 d using the optimized crystallization conditions (see below).

Commercial crystallization screening kits were used to determine likely conditions for crystal growth. Clusters of crystals with the shape of thin plates were observed from the initial screening conditions Hampton Research Crystal Screen HT condition E5 (2 M ammonium sulfate, 5% isopropanol), Emerald BioSystems Wizard I and II condition A8 (2 M ammonium sulfate, 0.1 M citrate pH 5.5) and Molecular Dimensions JCSG-plus conditions C11 (2 M ammonium sulfate, 0.1 M sodium acetate pH 4.6) and G11 (2 M ammonium sulfate, 0.1 M bis-tris pH 5.5). Subsequent optimization of various crystallization parameters led to final conditions consisting of 1.5 M ammonium sulfate, 0.1 M sodium acetate pH 4.2, 170 mM guanidine-HCl, 0.25% galactose, which reproducibly produced three-dimensional single crystals that grew to dimensions of 0.2 × 0.2 × 0.05 mm within

10–20 h (Fig. 2*a*). However, these crystals needed to be harvested before they overgrew into clusters; they were treated with a cryo-protectant consisting of crystallization well solution plus 28% glycerol before flash-cooling in liquid nitrogen.

Crystals of dscPsaA diffracted X-rays to 1.5 Å resolution (Fig. 2*b*) using a synchrotron-radiation source. For the native data set, 180° of data were collected with 1° oscillation per frame and a crystal-to-detector distance of 200 mm. The diffraction pattern revealed the symmetry of an orthorhombic lattice. A data set complete to 1.9 Å resolution was obtained with an R_{merge} of 8.6% (Table 1). The bromide-derivatized crystal diffracted to higher resolution. The same data-collection strategy was employed as for the native crystal except that the crystal-to-detector distance was set to 150 mm and the X-ray wavelength was tuned to the absorption edge of bromine (Table 1). A data set for the derivative crystal was obtained to 1.5 Å resolution with an R_{merge} of 11%.

Systematic absences were analyzed using *phenix.xtriage* from the *PHENIX* program package; diffraction conditions were only observed for (0, 0, l) reflections with $l = 2n$, thus establishing $P222_1$ space-group symmetry. The unit-cell parameters of the dscPsaA crystals were $a = 26.1$, $b = 54.2$, $c = 101.6$ Å. The Matthews coefficient (V_M) was calculated as 2.2 Å³ Da⁻¹, assuming the presence of one molecule per crystallographic asymmetric unit (Matthews, 1968).

3.2. Search for heavy-atom-derivatized dscPsaA crystals

Crystals of dscPsaA treated with various heavy-metal compounds such as mercury, lead and platinum compounds did not produce a derivative data set useful for phasing; this is possibly owing to the fact that the pilin subunit is an extracellular protein that is not expected to have free cysteine residues, to the presence of 1.5 M ammonium sulfate or to the very low pH in the crystallization conditions, all of which are known to interfere with binding of these heavy-metal compounds. To overcome this problem, we devised a crystal-stabilization solution that contained a high concentration (3 M) of sodium malonate, which allowed us to experiment with halide soaks (Dauter & Dauter, 2007). The halide soaking was performed at 277 K 1 h after the crystal had been treated in the stabilization solution by

(a)

(b)

(c)

Figure 2

Crystals of dscPsaA and their X-ray diffraction. (a) Crystals of dscPsaA. (b) An X-ray diffraction image obtained from a bromide-derivatized dscPsaA crystal at a synchrotron-radiation source. The diffraction resolution is about 1.4 Å at the edge of the square. (c) Experimental electron density computed using SIRAS phases. A section of the electron-density map is shown which is contoured at 1 σ and includes two bromide-binding sites (labeled).

Table 2
SIRAS phasing statistics for the structure determination of dscPsaA.

Bromide binding site	Br-atom position			Occupancy	B factor (Å ²)
	x	y	z		
1	22.686	51.962	8.588	1.00	2.8
2	2.710	29.8	5.158	1.00	15.1
3	4.565	45.954	17.718	0.67	7.0
4	9.791	38.447	15.335	0.81	11.9
5	21.316	0	0	0.45	7.7
6	5.904	22.595	1.656	0.49	6.5
7	8.597	8.239	4.116	0.42	8.8
8	11.714	32.261	15.326	0.48	5.2
9	3.775	13.294	19.054	0.38	10.0
10	23.219	7.031	23.156	0.32	3.4
11	18.819	21.799	2.264	0.25	7.8
12	14.161	29.780	3.944	0.17	5.6
13	5.939	41.816	4.703	0.20	4.9
14	5.097	53.335	24.234	0.22	5.6
15	14.954	34.973	19.966	0.20	7.5
16	23.718	33.516	22.876	0.26	6.5

Resolution shells (Å)	9.13	5.72	4.46	3.77	3.33	3.01	2.72	2.58	Overall
No. of centric reflections	129	146	147	126	138	134	145	139	1104
PP† for centric reflections	0.96	0.73	0.46	0.48	0.58	0.47	0.60	0.66	0.63
No. of acentric reflections	147	311	436	512	595	670	741	795	4207
PP† for acentric reflections	1.32	1.04	0.63	0.56	0.68	0.75	0.83	0.97	0.75
Mean figure of merit	0.45	0.40	0.34	0.36	0.40	0.43	0.46	0.47	0.42

† Phasing power is defined as $\langle f_H \rangle / \text{residual}$, where $\langle f_H \rangle$ is the root-mean-square (r.m.s.) of the structure-factor contribution in amplitudes from heavy atoms in the derivative and residual is the r.m.s. of the lack-of-closure error.

transferring the crystals to the soaking solution containing 1.5 M NaBr for 1 min.

3.3. Phase determination

Diffraction data sets from native and bromide-derivatized dscPsaA crystals were collected and their quality statistics are given in Table 1. A native data set was processed to 1.9 Å resolution with an R_{merge} of 8.6% and a completeness of 97.7%. The bromide-derivatized crystal diffracted to better than 1.45 Å resolution and was processed to 1.5 Å resolution with a merging R factor of 13.6%. Both data sets were required for successful SIRAS phasing. 16 bromide-binding sites were found for the derivative data set using the PHENIX package,

giving rise to an overall figure of merit of 0.42 in the resolution range 9.13–2.58 Å (Table 2). After phase improvement by density modification, the resulting experimental electron density clearly contained recognizable features for protein molecules and was amenable for the automated chain-tracing procedure. Residues with heavy atoms bound were unambiguously identified and were helpful in sequence assignment in subsequent model building, which is currently in progress. Based on the electron-density map, there is one molecule in the crystallographic asymmetric unit.

The authors wish to thank the beamline staff of SER-CAT at APS, ANL for their assistance in data collection. We thank George Leiman for editorial assistance during the preparation of the manuscript. This research was supported in part by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research, by a grant from the Trans NIH/FDA Intramural Biodefense Program (to DX), by NIH grant AI076695, by a University of Pennsylvania Research Foundation grant and by Research Initiative Funds from the University of Pennsylvania Veterinary Center for Infectious Disease (to DMS).

References

Adams, P. D. *et al.* (2010). *Acta Cryst.* **D66**, 213–221.
Ben-Efraim, S., Aronson, M. & Bichowsky-Slomnicki, L. (1961). *J. Bacteriol.* **81**, 704–714.
Dauter, M. & Dauter, Z. (2007). *Methods Mol. Biol.* **364**, 149–158.
Galván, E. M., Chen, H. & Schifferli, D. M. (2007). *Infect. Immun.* **75**, 1272–1279.
Huang, X.-Z. & Lindler, L. E. (2004). *Infect. Immun.* **72**, 7212–7219.
Lindler, L. E. & Tall, B. D. (1993). *Mol. Microbiol.* **8**, 311–324.
Liu, F., Chen, H., Galván, E. M., Lasaro, M. A. & Schifferli, D. M. (2006). *Infect. Immun.* **74**, 5636–5644.
Ma, J. & Xia, D. (2008). *J. Appl. Cryst.* **41**, 1150–1160.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 499–501.
Nuccio, S. P. & Bäuml, A. J. (2007). *Microbiol. Mol. Biol. Rev.* **71**, 551–575.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Parkhill, J. *et al.* (2001). *Nature (London)*, **413**, 523–527.
Payne, D., Tatham, D., Williamson, E. D. & Titball, R. W. (1998). *Infect. Immun.* **66**, 4545–4548.
Yang, Y., Merriam, J. J., Mueller, J. P. & Isberg, R. R. (1996). *Infect. Immun.* **64**, 2483–2489.