Surface-layer protein from *Caulobacter crescentus*: expression, purification and X-ray crystallographic analysis

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Protein surface layers are self-assembling, paracrystalline lattices on the surface of many prokaryotes. Surface-layer proteins have not benefited from widespread structural analysis owing to their resistance to crystallization. Here, the successful expression of a truncated version of RsaA, the surface-layer protein from *Caulobacter crescentus*, from a *Caulobacter* protein-expression system is reported. The purification, crystallization and initial X-ray diffraction analysis of the truncated RsaA, the largest surface-layer protein studied to date and the first from a Gram-negative bacterium, are also reported.

1. Introduction

Protein surface layers (S-layers) are paracrystalline coatings that surround microbial cells (Beveridge *et al.*, 1997). They have various functions, such as adhesion and immune evasion in pathogenic bacteria (Dallaire-Dufresne *et al.*, 2014; Kern & Schneewind, 2010), protection against phages and predation (Koval, 1993; Koval & Hynes, 1991), and as a pseudo-membrane in archaea (Blaurock *et al.*, 1976). S-layers are composed of either one or a small number of secreted proteins that self-assemble into a contiguous sheet in the extracellular environment. Despite their unique features, S-layers have not enjoyed the intensive structural investigations that most other protein families have received, in no small part owing to their proclivity to self-assemble into large two-dimensional polymeric structures that resist crystallization.

The Gram-negative bacterium *Caulobacter crescentus* possesses an S-layer comprised of a single 98.6 kDa protein, RsaA (Smit *et al.*, 1981). This particular S-layer has been pursued as a potent platform for peptide display and protein expression (Smit, 2008). RsaA is secreted from the cell *via* a type I secretion system (Awram & Smit, 1998). RsaA shares little to no sequence similarity with any other known protein, except for in the conserved RTX (repeat-in-toxin) motifs that are canonically found in all type I secreted proteins (Chenal *et al.*, 2009; Bingle *et al.*, 2000). RsaA further isolates itself in its sequence by featuring a uniquely limited amino-acid composition that is biased towards small, simple residues. This bias towards amino acids that are energetically less costly to synthesize is thought to be an evolutionary strategy for secreted proteins that is conserved across microbial life and is notably present in *C. crescentus* (Smith & Chapman, 2010).

It may be that all crystallographic studies of S-layer proteins must proceed by inhibiting S-layer formation. In the past,

Table 1

X-ray data-collection statistics for RsaA $_{\Delta 0-222}$ processed with *HKL-3000* (Minor *et al.*, 2006).

Values in parentheses are for the highest resolution shell.

Space group	$P2_1$
Unit-cell parameters ($\text{\AA}$, $^\circ$)	$a = 210.7$, $b = 80.7$, $c = 221.9$, $\beta = 117.5$
Resolution ($\text{\AA}$)	50.00–2.45 (2.51–2.45)
R_{merge}	0.11 (0.68)
$\langle I/\sigma(I) \rangle$	9.9 (1.8)
Maximum-likelihood Wilson B factor ($\text{\AA}^2$)	25.1
Unique reflections	475807
Completeness (%)	99.7 (99.8)
Multiplicity	3.2
$CC_{1/2}$	99.5 (80.1)

co-crystallization with nanobodies (Baranova *et al.*, 2012) and truncation (Pavkov *et al.*, 2008) approaches have resulted in monodisperse protein samples that are suitable for crystallization. Here, we describe the expression of an 804-amino-acid C-terminal fragment of RsaA (RsaA $_{\Delta 0-222}$) from its native host, *C. crescentus*. The untagged protein was purified from the culture supernatant and crystallized by hanging-drop vapor diffusion. Diffraction data from RsaA $_{\Delta 0-222}$ crystals were collected at the Canadian Light Source (CLS) to ~ 2.5 $\text{\AA}$ resolution. We are currently unable to phase the data, but the use of iodine soaking has proved promising. Here, we report our progress on the structural study of RsaA $_{\Delta 0-222}$, which represents both the first Gram-negative S-layer protein to be crystallized and the largest S-layer protein to be crystallized to date.

2. Materials and methods

2.1. Macromolecule production

The plasmid used in this study, pUC8CVX-RsaA $_{\Delta 0-222}$, is a construct of the plasmid pUC8CVX (Nomellini *et al.*, 2007)

containing an open reading frame encoding the C-terminal 804 amino acids of RsaA from *C. crescentus*. pUC8CVX, digested with BamHI and HindIII, was ligated to the 2819 bp fragment from a similarly digested plasmid containing an open reading frame encoding RsaA with a BamHI endonuclease recognition sequence at the 666 bp position created in a previous study (Ford *et al.*, 2007). The plasmid, pUC8CVX-RsaA $_{\Delta 0-222}$, was then electroporated (Gilchrist & Smit, 1991) into the expression strain *C. crescentus* JS1014 (Ford *et al.*, 2007). JS1014 is a protein-expression strain that has had its genomic copy of *rsaA* and a surface-associated metallo-protease, *sapA*, knocked out; SapA has previously been found to degrade modified versions of RsaA (Ford *et al.*, 2007). In addition, all major surface polysaccharides are eliminated by knockout of the *manB* gene, which prevented any possibility of RsaA surface attachment and minimized cell buoyancy.

The cells were grown in M16 medium (Jones *et al.*, 2015) at 30°C for 72 h. 250 ml cultures were grown in 2500 ml wide-bottom Fernbach flasks, resulting in shallow cultures that did not require shaking for sufficient aeration, as agitation of the culture led to macroaggregation of the secreted protein. The cultures were centrifuged at 6300 rev min $^{-1}$ ($\sim 7000g$) in Beckman JA-10 bottles for 35 min and the supernatants were recovered and recentrifuged. The supernatants were then filtered (0.22 μm) to ensure that no cells remained. The protein-containing supernatant was initially concentrated by lyophilization and rehydration to one tenth of the original volume. The rehydrated protein solution was dialyzed against distilled water and filtered (0.22 μm). Purification was performed on a 26/60 Sephadex S-100 size-exclusion column with a mobile phase consisting of 200 mM NaCl, 10 mM Tris pH 7.5. The first major elution peak was pooled and dialyzed against distilled water and concentrated by dry dialysis against high-molecular-weight polyethylene glycol (PEG 20M), slowly concentrating to a final concentration of 3.5–4 mg ml $^{-1}$.

2.2. Crystallization

RsaA $_{\Delta 0-222}$ was initially crystallized in 200 mM CaCl $_2$, 100 mM Tris-HCl pH 7, 20% PEG 8000 from a sparse-matrix screen (The JCSG Core I Suite, Qiagen) prepared in a 96-well sitting-drop plate. Small, thin crystals were observed after one week of incubation at room temperature. However, these crystals were not reproducible in hanging-drop vapor-diffusion wells. Larger, but still thin and delicate, plates readily formed through optimization of both the protein and precipitant concentration. Thicker and more robust crystals were produced when CaCl $_2$ was substituted with other group 2 alkaline-earth metal salts, specifically SrCl $_2$ or BaCl $_2$. SrCl $_2$ was subsequently chosen based on overall crystal quality. Use of MgCl $_2$ was attempted, but produced thinner plates. The final crystals were grown by hanging-drop vapor diffusion in 8.5% PEG 8000, 100 mM Tris-HCl pH 7.4, 150 mM SrCl $_2$ over 2–3 weeks at room temperature using a starting protein concentration of 3.5 mg ml $^{-1}$ and were shown to consist of RsaA $_{\Delta 0-222}$ using SDS-PAGE (Fig. 1).

Figure 1

SDS-PAGE gels of purified soluble RsaA $_{\Delta 0-222}$ (left) and a dissolved crystal of RsaA $_{\Delta 0-222}$ (right) stained with Coomassie Blue. Molecular masses are labelled in kDa.

2.3. Data collection and processing

Crystals were briefly soaked in mother liquor containing 25% glycerol as a cryoprotectant and 0.5–1.0 M potassium iodide prior to flash-cooling in liquid nitrogen. Diffraction data were then collected at a wavelength of 1.54 Å on beamline 08ID-1 at the Canadian Light Source in Saskatoon, Saskatchewan to maximize the overall anomalous signal of iodide. Data were processed using *HKL-3000* (Minor *et al.*, 2006) or *XDS* (Kabsch, 2010) using a script developed at the Canadian Light Source (Table 1); phasing attempts were performed using *SHELX* (Sheldrick, 2008) or *AutoSol* in *PHENIX* (Adams *et al.*, 2010).

3. Results and discussion

RsaA $_{\Delta 0-222}$ was expressed using our *C. crescentus* protein-expression system (Bingle *et al.*, 1997; Umelo-Njaka *et al.*, 2001; Bingle & Smit, 1990). All attempts to produce protein in *Escherichia coli* resulted in highly insoluble inclusion bodies that resisted refolding after solubilization with 6 M guanidine-HCl (data not shown). Expression levels of RsaA in its native host are notably high (Lau *et al.*, 2010), but the problem of aggregation persisted for the secretion of full-length RsaA by *Caulobacter*. The problem was assuaged by truncating the N-terminus, producing RsaA $_{\Delta 0-222}$, and was markedly improved by culturing the cells without agitation in wide, shallow flasks. Our shallow-culture expression system produced 130–150 mg purified, concentrated protein per litre of culture supernatant. Although RsaA is the natural product of *C. crescentus*, this system is easily amenable to producing exogenous protein for crystallization by simple fusion to the required type 1 secretion signal (Bingle *et al.*, 2000).

The reasons for using an N-terminal truncation of RsaA were twofold: the N-terminus anchors RsaA to the outer membrane *via* a lipopolysaccharide O side chain and it has

been identified as the center of threefold symmetry in the assembled S-layer (Amat *et al.*, 2010). Therefore, its deletion results in soluble protein that no longer leads to wide-scale S-layer formation or aggregation and is freely secreted into the supernatant. The success of structurally characterizing S-layer proteins depends upon overcoming such issues and our strategies resulted in large well diffracting crystals (Fig. 2).

The ‘plate’ crystals diffracted to ~ 2.5 Å resolution, with unit-cell parameters $a = 210.7$, $b = 80.7$, $c = 221.9$ Å, $\beta = 117.5^\circ$. These unit-cell sizes closely match the two-dimensional unit size of the native S-layer repeating unit, a hexamer of RsaA monomers (Smit *et al.*, 1992). These results suggest that the asymmetric unit contains at least six copies of RsaA $_{\Delta 0-222}$, corresponding to a solvent content of $\sim 60\%$. The X-ray diffraction patterns exhibited anisotropy in one axis (see Fig. 3). As our protein has no close matches in structural databases to use for the purpose of molecular replacement, iodine derivatization was attempted. Patterson analysis of the data set suggests issues with pseudo-translational symmetry confounding phasing attempts (data not shown). The largest off-origin Patterson peak has a height of $\sim 30\%$ (p -value = 1.8×10^{-3}) and is situated at (0.055, 0.500, 0.009) in fractional coordinates. A self-rotation analysis reveals strong peaks along the y axis for $\chi = 60$ and 120° , supporting a hypothesis for the presence of sixfold noncrystallographic symmetry and a hexamer of RsaA $_{\Delta 0-222}$. Strong peaks are also observed for $\chi = 180^\circ$ along y , as expected for the crystallographic twofold symmetry of $P2_1$. For $\chi = 180^\circ$, a weaker peak is also observed near the x axis, which may suggest pseudo-symmetry between the top and bottom halves of the S-layer core. Twinning analyses (Adams *et al.*, 2010) indicate that no twinning is suspected. Attempts to use bromine or lanthanides for anomalous phasing are in progress.

Figure 2

A plate-shaped crystal of RsaA $_{\Delta 0-222}$ that formed after three weeks of crystallization by hanging-drop vapor diffusion at ambient temperature. The scale bar indicates 0.5 mm.

Figure 3

A representative X-ray diffraction image of the RsaA $_{\Delta 0-222}$ crystal.

Solving the crystal structure of RsaA will provide insights into the self-assembly mechanisms of S-layer proteins. Because the *C. crescentus* S-layer is being developed for exogenous peptide-display applications, an atomic scale structure of RsaA will aid in the rational design of future biotechnology constructs.

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