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Crystallization and preliminary X-ray diffraction studies of sortase A from *Streptococcus pneumoniae*

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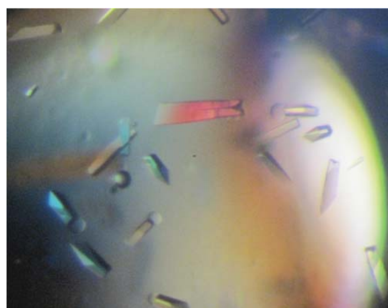
Sortases are cell-membrane-anchored cysteine transpeptidases that are essential for the assembly and anchoring of cell-surface adhesins in Gram-positive bacteria. Thus, they play critical roles in virulence, infection and colonization by pathogens. Sortases have been classified into four types based on their primary sequence and the target-protein motifs that they recognize. All Gram-positive bacteria express a class A housekeeping sortase (SrtA). Sortase A from *Streptococcus pneumoniae* (NP_358691) has been crystallized in two crystal forms. Diamond-shaped crystals of ΔN_{59}SrtA diffracted to 4.0 Å resolution and belonged to a tetragonal system with unit-cell parameters $a = b = 122.8$, $c = 86.5$ Å, $\alpha = \beta = \gamma = 90^\circ$, while rod-shaped crystals of ΔN_{81}SrtA diffracted to 2.91 Å resolution and belonged to the monoclinic space group $P2_1$ with unit-cell parameters $a = 66.8$, $b = 103.47$, $c = 74.79$ Å, $\alpha = \gamma = 90$, $\beta = 115.65^\circ$. The Matthews coefficient ($V_M = 2.77$ Å^3 Da^{-1}) with ~56% solvent content suggested the presence of four molecules in the asymmetric unit for ΔN_{81}SrtA. Also, a multi-copy search using a monomer as a probe in the molecular-replacement method resulted in the successful location of four sortase molecules in the asymmetric unit, with statistics $R = 41.61$, $R_{free} = 46.44$, correlation coefficient (CC) = 64.31, $CC_{free} = 57.67$.



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1. Introduction

Pneumococcal diseases caused by *Streptococcus pneumoniae* lead to approximately 1.6 million deaths annually, including those of one million children under the age of five (Levine *et al.*, 2006). The increasing prevalence of antibiotic-resistant strains necessitates the development of novel therapeutics. Sortase A (EC 3.4.22.70) is an attractive drug target because it is present in all Gram-positive bacteria (Cossart & Jonquières, 2000) and there are no sortase-related homologues in eukaryotes. Sortase A is important for cell-surface anchoring of several virulence proteins to the bacterial cell-wall peptidoglycan (Hendrickx *et al.*, 2011). Sortase A substrates are characterized by the presence of a C-terminal cell-wall-sorting signal, which comprises of a sortase-recognition motif LP*X*TG followed by a stretch of hydrophobic residues and a positively charged tail (Schneewind *et al.*, 1993). Knocking out sortase impacts pathogenesis but has no effect on viability. Therefore, inhibition of sortase would reduce the selection pressure towards the development of drug resistance (Mazmanian *et al.*, 1999). The presence of sortase in the cell membrane rather than inside the cell makes it easily accessible to inhibitors (Clancy *et al.*, 2010). Additionally, sortases have important biotechnological applications (Samantaray *et al.*, 2008; Proft, 2010). To date, crystal structures of sortase A from *Staphylococcus aureus* (Zong *et al.*, 2004) and from *Streptococcus pyogenes* (Race *et al.*, 2009) have been determined. Sortase A from both these organisms adopts an eight-stranded β-barrel structure. The overall fold is conserved among the sortase isoforms, with some modifications (Clancy *et al.*, 2010).

This paper reports the crystallization and preliminary structural analysis of sortase A from *Streptococcus pneumoniae*. The full-length *S. pneumoniae* sortase A is more closely related to *S. pyogenes* sortase A than to *S. aureus* sortase A in terms of sequence identity (63% *versus* 28% for sequence-alignment lengths of 166 and 118, respectively). The structure of the protein would aid in the under-

standing of its mechanism of catalysis and substrate recognition and eventually in the design of structure-based inhibitors.

2. Materials and methods

2.1. Cloning, expression and purification of sortase A

There are intrinsic difficulties in the expression and purification of full-length sortases because they are membrane-anchored proteins. Deletion of the transmembrane region at the N-terminus facilitates the expression of soluble protein (Ilangovan *et al.*, 2001). The DNA encoding residues 60–247 of *S. pneumoniae* sortase A (ΔN_{59}SrtA), analogous to the *S. aureus* ΔN_{59}SrtA construct, was amplified from genomic DNA of *S. pneumoniae* strain R6 by PCR using gene-specific primers. The amplified fragment was digested with *Nde*I and *Hind*III and cloned into a pET28c expression vector. The vector adds an N-terminal hexahistidine tag (MGSSHHHHHHSSGLVPRSHM) to the expressed protein. The pET28c-ΔN_{59}SrtA construct was transformed into *Escherichia coli* BL21 (DE3) cells for overexpression of the recombinant protein. The cells were grown at 310 K until the OD_{600} reached 0.6; they were then induced with 0.5 m*M* IPTG and grown for a further 5 h at 303 K prior to harvesting. The harvested cells were resuspended in buffer *R* (10 m*M* Tris pH 7.5, 40 m*M* NaCl, 2 m*M* β-mercaptoethanol) and lysed by sonication. The cell lysate was centrifuged at 12 000 rev min^{-1} for 30 min and the resulting supernatant was loaded onto an Ni–NTA agarose column which had been equilibrated with five column volumes of buffer *R*. Upon binding of the recombinant protein to the Ni–NTA agarose, the column was washed with ten column volumes of a buffer consisting of 10 m*M* Tris pH 7.5, 30 m*M* imidazole, 500 m*M* NaCl and 2 m*M* β-mercaptoethanol. The His-tagged protein was eluted with five column volumes of buffer *R* containing 250 m*M* imidazole. The eluted protein was concentrated using Millipore Amicon filters (10 kDa molecular-weight cutoff) and loaded onto a PD-10 column (GE Healthcare, USA) for desalting. The purified protein was analyzed on SDS–PAGE.

The *S. pneumoniae* ΔN_{81}SrtA construct (residues 82–247), which is analogous to the *S. pyogenes* ΔN_{81}SrtA construct, was subcloned from the pET28c-ΔN_{59}SrtA construct into pET28c vector. The pET28c-ΔN_{81}SrtA construct was transformed into *E. coli* BL21 (DE3) cells. The *S. pneumoniae* ΔN_{81}SrtA protein was purified using the same protocol as followed for *S. pneumoniae* ΔN_{59}SrtA.

2.2. Crystallization

Initial screening for crystallization conditions for the ΔN_{59}SrtA protein construct (~23 kDa) was performed using commercially available crystallization solutions. The conditions were examined using the hanging-drop method at 293 K; the drop (4 µl) consisted of 2 µl protein solution (5–15 mg ml^{-1} in 10 m*M* Tris buffer pH 7.5) and 2 µl reservoir solution. The ΔN_{59}SrtA construct containing an N-terminal His tag crystallized as diamond-, needle-, rod- and wedge-shaped crystal forms (Fig. 1) in more than one crystallization condition within two to four weeks. The crystals grew to their maximum size in a month. However, these crystals did not diffract either at the home source or the synchrotron.

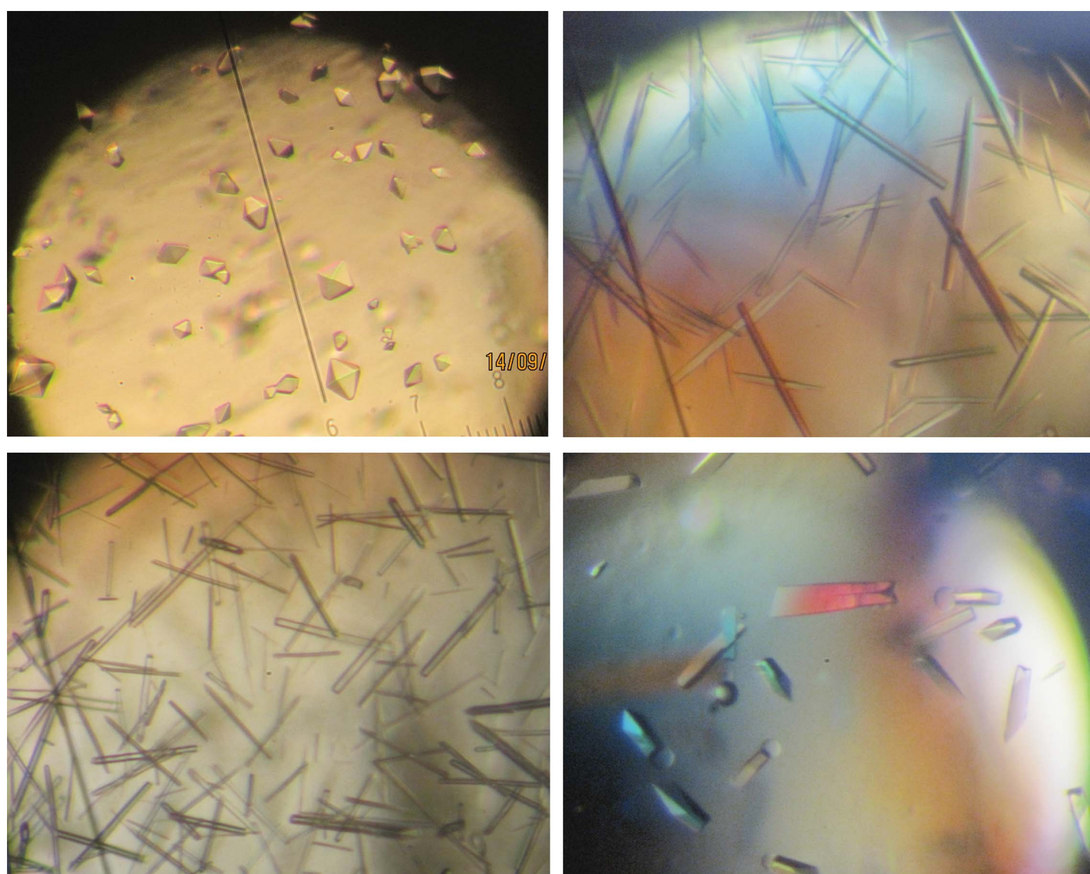

Figure 1
ΔN_{59}SrtA crystals grown under different crystallization conditions using the microbatch-under-oil method resulted in different forms (diamond-shaped, rod-shaped, needle-shaped and wedge-shaped).

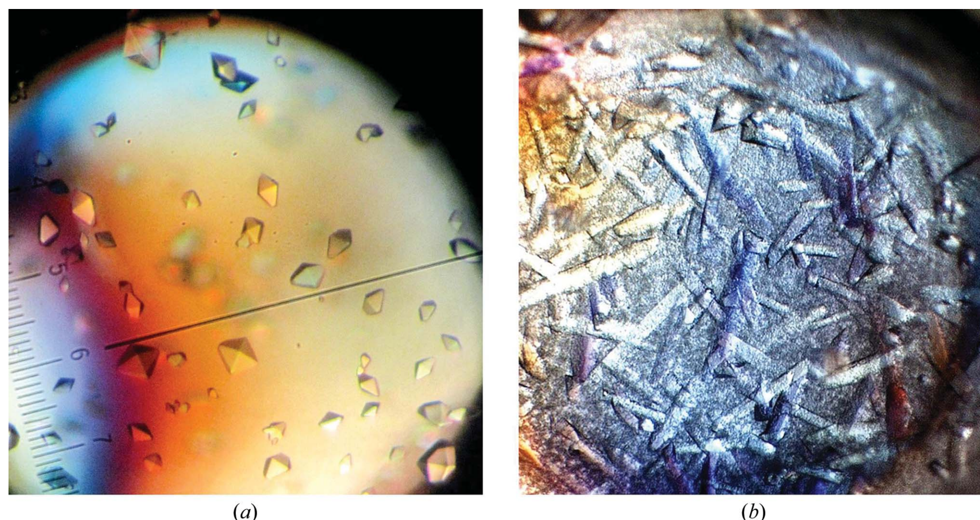

Figure 2
Crystals of sortase A grown using the microbatch-under-oil method. (*a*) ΔN_{59}SrtA crystals belonging to the tetragonal system with dimensions ~0.2 × 0.2 × 0.1 mm. (*b*) ΔN_{81}SrtA crystals belonging to the monoclinic space group $P2_1$ with dimensions ~0.5 × 0.1 × 0.05 mm.

In another attempt at crystallization, trials were set up in microbatch plates at 293 K using 2 µl protein sample (6 mg ml^{-1} in 10 m*M* Tris buffer pH 7.5) and 2 µl crystallization condition under a 1:1 mixture of silicon oil and paraffin oil. Diamond-shaped crystals again appeared within two weeks in a solution consisting of 1.1 *M* sodium malonate and 0.1 *M* HEPES pH 7.0. These crystals (Fig. 2*a*) diffracted to 4.0 Å resolution at the home source.

Subsequently, we made modifications to the SrtA construct in order to improve the diffraction quality of the crystals. For further crystallization experiments, we used the new construct ΔN_{81}SrtA, containing an N-terminal His tag, which gave well diffracting rod-shaped crystals at a protein concentration of 25 mg ml^{-1} (in 10 m*M* Tris buffer pH 7.5) with a solution of 0.2 *M* tri-ammonium citrate and 20%(*w*/*v*) PEG 3350 pH 7.0 using the microbatch-under-oil crystallization method (Fig. 2*b*).

2.3. Data collection and processing

Crystals suitable for data collection were flash-cooled in liquid nitrogen. Data were collected from diamond-shaped ΔN_{59}SrtA crystals of dimensions ~0.2 × 0.2 × 0.1 mm and rod-shaped ΔN_{81}SrtA crystals of dimensions ~0.5 × 0.1 × 0.05 mm at 100 K (Oxford Cryosystems) using a MAR imaging-plate system (diameter 345 mm) mounted on a Bruker Microstar Ultra 2 rotating-anode generator (Cu $K\alpha$ radiation; λ = 1.5418 Å). Crystals of the ΔN_{59}SrtA construct diffracted to 4.0 Å resolution (data not presented). None of the known cryoprotectants were found to improve the diffraction quality. Crystals of the modified construct ΔN_{81}SrtA diffracted to 2.91 Å resolution without using any external cryoprotectant apart from the 20% PEG 3350 present in the crystallization condition (Fig. 3). A total of 408 frames were collected with 5 min exposure per frame, 0.5° oscillation and a crystal-to-detector distance of 180 mm.

The intensity data were processed using *MOSFLM* (Leslie, 2006) and were scaled using the program *SCALA* (Evans, 1993) from the *CCP*4 program suite (Winn *et al.*, 2011). Structure-factor amplitudes were obtained from intensities using *TRUNCATE* (French & Wilson, 1978) from the *CCP*4 suite. Data-collection and processing statistics for the ΔN_{81}SrtA crystal are presented in Table 1.

3. Results and discussion

Diamond-shaped crystals of ΔN_{59}SrtA appeared two to four weeks after setting up crystallization experiments using the microbatch-under-oil method. These crystals (Fig. 2*a*) had dimensions of ~0.2 × 0.2 × 0.1 mm and diffracted to 4.0 Å resolution. The ΔN_{59}SrtA crystals belonged to the tetragonal system, with unit-cell parameters $a = b = 122.8$, $c = 86.5$ Å, $\alpha = \beta = \gamma = 90°$. To overcome the poor diffraction quality of the crystals obtained using this construct, we crystallized the construct ΔN_{81}SrtA with a rod-shaped crystal morphology with approximate dimensions of 0.5 × 0.1 × 0.05 mm; the crystals belonged to the monoclinic space group $P2_1$ (Fig. 2*b*). The crystals appeared in about two weeks and had unit-cell parameters $a = 66.8$, $b = 103.47$, $c = 74.79$ Å, $\alpha = \gamma = 90$, $\beta = 115.65°$. ΔN_{81}SrtA

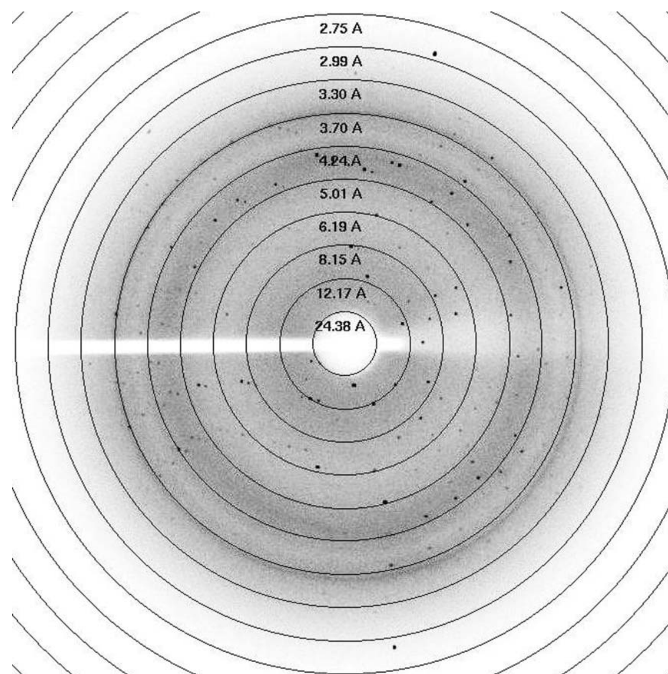

Figure 3
Diffraction pattern of the ΔN_{81}SrtA crystal.

Table 1
Summary of data-collection and processing statistics for the ΔN_{81}SrtA crystal.

Values in parentheses are for the last resolution shell.

Resolution range (Å)	30.05–2.91 (3.07–2.91)
Crystal system	Monoclinic
Space group	$P2_1$
Unit-cell parameters	
a (Å)	66.8
b (Å)	103.47
c (Å)	74.79
β (°)	115.65
Mosaicity (°)	0.75
Total No. of observed reflections	85159 (12291)
Unique reflections	20193 (2920)
Multiplicity	4.2 (4.2)
Mean $I/\sigma(I)$	10.9 (2.9)
Completeness (%)	99.9 (99.9)
$R_{\rm merge}$† (%)	10.7 (49.1)
Overall B factor from Wilson plot (Å^2)	42.0
No. of molecules in the asymmetric unit	4
$V_{\rm M}$ (Å^3 Da^{-1})	2.77
Solvent content (%)	56

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the ith observation of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity over all observations.

only crystallized with a rod-like morphology, whereas ΔN_{59}SrtA crystallized with four different morphologies. The data-collection statistics for the rod-shaped ΔN_{81}SrtA crystal are reported in Table 1 and a diffraction pattern is shown in Fig. 3. Based on the molecular weight and the space group, it was inferred that there were four molecules of ΔN_{81}SrtA in the asymmetric unit of the crystal, which corresponds to a solvent content of about 56% (Matthews, 1968). Initial molecular-replacement trials were performed in *Phaser* (Read, 2001; Storoni *et al.*, 2004) using the sortase A protein from *S. pyogenes* (PDB entry 3fn5; Race *et al.*, 2009), which has 64% sequence identity over 164 residues, as a search model. The best solution using *Phaser* (TFZ score = 7.5, LLG score = 930), as well as the initial R and $R_{\rm free}$ after the first refinement cycle (41.61 and 46.44, respectively, with correlation coefficients CC = 64.31 and CC$_{\rm free}$ = 57.67), supports the presence of four sortase subunits in the asymmetric unit. These four molecules are arranged tetrahedrally (Katre & Suresh, 2009) and can also be described as a dimer of dimers oriented nearly perpendicular to each other at their ends. In this regard, it is pertinent to note that the prototypical sortase A from *S. aureus* displays a strong tendency to form oligomers and exists predominantly as a dimer (Lu *et al.*, 2007). The dimeric enzyme shows enhanced catalytic activity. It is conceivable that *S. pneumoniae* sortase A (ΔN_{81}SrtA) is also endowed with similar attributes. Crystal structure determination of ΔN_{81}SrtA is in progress. The determined structure is likely to shed light on sortase catalysis as well as to help in the structure-based design of sortase inhibitors.

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