Acta Crystallographica Section F
Structural Biology and Crystallization Communications
ISSN 1744-3091

Cloning, expression, purification, crystallization and preliminary X-ray analysis of the pilus-associated sortase C from *Streptococcus pneumoniae*

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Received 29 October 2008
Accepted 27 November 2008

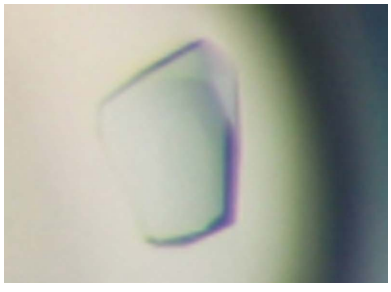
The pilus-associated sortase C from *Streptococcus pneumoniae* (SrtC or Srt-2) acts as a polymerase for the pilus subunit proteins RrgA and RrgB. Here, the crystallization and preliminary X-ray diffraction analysis of three crystal forms of SrtC are reported. One crystal form belongs to space group $P2_12_12_1$, with unit-cell parameters a = 48.9, b = 96.9, c = 98.9 Å, $\alpha = \beta = \gamma = 90^\circ$. The other two crystal forms belong to space group $P222$, with unit-cell parameters a = 48.8, b = 97.2, c = 99.2 Å, $\alpha = \beta = \gamma = 90^\circ$ and a = 48.6, b = 96.5, c = 98.8 Å, $\alpha = \beta = \gamma = 90^\circ$, respectively. Preliminary analysis indicates the presence of two molecules in the asymmetric unit of the crystal for all three forms.

1. Introduction

Pneumonia is one of the leading single causes of mortality in children aged less than 5 y (Williams *et al.*, 2002). One of the main pathogens associated with pneumonia is the Gram-positive human pathogen *Streptococcus pneumoniae* (pneumococcus). Recently, we have described filamentous pilus structures extending from the surface of this bacterium (Barocchi *et al.*, 2006). Similar to pili from other Gram-positive organisms, these pili play an important role in adhesion to host cells (Mandlik *et al.*, 2007; Nelson *et al.*, 2007). The pneumococcal pilus is encoded by the *rlrA* genetic islet. This islet encodes the positive regulator RlrA, the three structural subunits RrgA, RrgB and RrgC and the three sortase enzymes SrtB, SrtC and SrtD. Housekeeping sortases have been extensively studied and have been shown to transfer surface proteins to the peptidoglycan cell wall (Marraffini *et al.*, 2006). In contrast, pilus-associated sortases have been implicated to act as transpeptidases that covalently link the pilus subunits to each other (Budzik *et al.*, 2007; LeMieux *et al.*, 2008).

In pneumococci, RrgB is the major pilin and forms the backbone of the pili. RrgA and RrgC are linked to the RrgB backbone, thereby decorating the pilus shaft (Hilleringmann *et al.*, 2008). Recently, the relative contributions of the three sortases to incorporation of the subunits have been elucidated (Falker *et al.*, 2008). SrtB (or Srt-1) and SrtC (or Srt-2) show redundancy in terms of activity; both are able to polymerize RrgB and to link RrgA to the backbone. However, in contrast to SrtB, SrtC is not able to incorporate RrgC. SrtD (or Srt-3) was not found to polymerize any of the subunits, but both SrtB and SrtD affect the surface localization of pili. SrtC has 49% and 21% sequence identity to SrtB and SrtD, respectively. Considering the functional differences observed between these three sortases, the determination of the three-dimensional structure of each sortase is necessary in order to assess the molecular basis underlying the production and formation of the pilus in *S. pneumoniae*.

Here, we report the cloning, production, purification, crystallization and preliminary diffraction data of SrtC. Three different crystals were obtained using both the sitting-drop and hanging-drop vapour-diffusion methods. The three crystals belonged to different



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space groups and diffracted to resolutions ranging between 2.3 and 1.7 Å.

2. Materials and methods

2.1. Cloning and expression

The DNA encoding residues 46–257 (SP_0467) of the SrtC protein was amplified from genomic DNA of *S. pneumoniae* strain TIGR4 using the primers 5′-CATATGTCAAACGAGGTTATTAAAGA-GTTTGATGAGACGG-3′ and 5′-GAGCTCTTAGAATTGCCCA-CGCTCTCTCACCGCTCG-3′. The DNA was incorporated into the pET24c plasmid (Novagen) by using *Nde*I and *Sac*I restriction sites. The resulting plasmid was transformed into *Escherichia coli* BL21 (DE3) (Novagen) for overexpression. Single colonies from a Luria–Bertani–kanamycin agar plate (containing 100 mg l^{-1} kanamycin) were used to inoculate a 100 ml starter culture (LB–kanamycin, containing 100 mg l^{-1} kanamycin), which was grown at 310 K overnight. 20 ml aliquots of the overnight culture were used to inoculate 1 l LB–amp broth. Expression was induced by the addition of 100 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG) to cultures with an OD_{600} of 0.6. The cultures were maintained at 310 K for 12 h following induction and the cells were harvested by centrifugation.

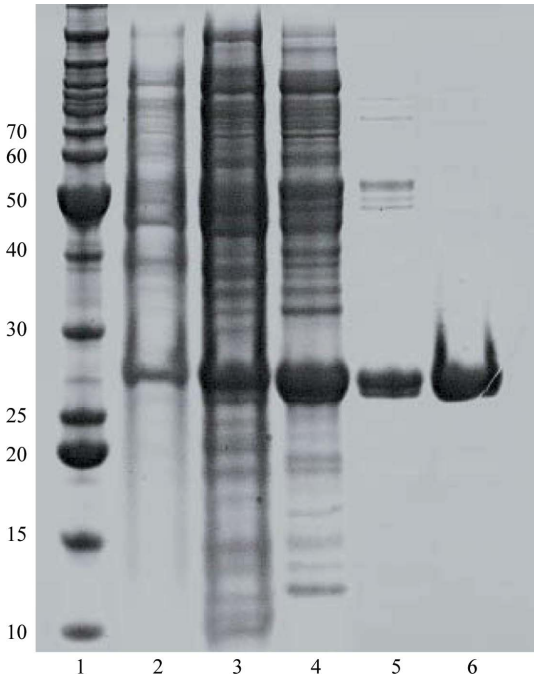

Figure 1
SDS–PAGE analysis of purified SrtC. Molecular-weight markers (in kDa; BenchMark from Invitrogen), the full cell contents prior to purification, the contents of the supernatant following passage through a French press and centrifugation, the DEAE Sepharose pooled fractions, the Phenyl-Sepharose pooled fractions and Superdex 75 pooled fractions are presented in lanes 1, 2, 3, 4 and 5, respectively.

2.2. Purification

Cells were resuspended in lysis buffer (50 m*M* Tris–HCl, 1 m*M* EDTA, 5 m*M* dithiothreitol), disrupted by two passages through a French press at room temperature and centrifuged at 17 000*g* for 45 min at 277 K. The supernatant cell lysate was loaded onto a 100 ml DEAE-Sepharose column (GE Healthcare). The column was washed with two column volumes of lysis buffer followed by a linear gradient of potassium chloride (0–1.0 *M*). The SrtC protein eluted at 0.25 *M* KCl. The fractions containing SrtC were pooled and the concentration of ammonium sulfate in the pooled fractions was slowly adjusted to 1 *M*. The pooled sample was applied onto a Phenyl-Sepharose column (GE Healthcare) preliminarily equilibrated with buffer *A* (50 m*M* Tris–HCl, 1 m*M* EDTA, 5 m*M* dithiothreitol, 1 *M* ammonium sulfate). The column was washed with two column volumes of buffer *A* followed by a linear gradient of lysis buffer in order to decrease the concentration of ammonium sulfate from 1.0 to 0 *M*. SrtC eluted at 0.2 *M* ammonium sulfate. The fractions containing SrtC were pooled, concentrated to 10 mg ml^{-1} using an Amicon Ultra with a cutoff of 10 kDa (Millipore) and finally applied onto a Superdex 75 column (isocratic gradient with 50 m*M* Tris–HCl, 1 m*M* EDTA). The DEAE Sepharose, Phenyl-Sepharose and Superdex 75 columns were connected to an ÄKTA FPLC (Amersham). The final fractions were concentrated to 10 mg ml^{-1}. Purity was checked by electrophoresis on 10% SDS–PAGE and by electrospray mass-spectrometric analyses. The molecular concentration was determined spectrophotometrically using an extinction coefficient of 17 420 M^{-1} cm^{-1} at 280 nm.

2.3. Crystallization and data collection

Following dialysis of SrtC against 50 m*M* Tris–HCl, 1 m*M* EDTA in order to remove dithiothreitol, screening assays were set up to identify the optimal crystallization conditions for SrtC (10 mg ml^{-1}) using a Phoenix crystallization robot (Art Robbins Instruments). The sitting-drop vapour-diffusion method was used in combination with sparse-matrix screens (Hampton Research, California, USA), PACT and JCSG+ (Qiagen) in 96-well Corning plates at 277 and 293 K. Nanodrops containing 0.1 µl protein solution mixed with 0.1 µl

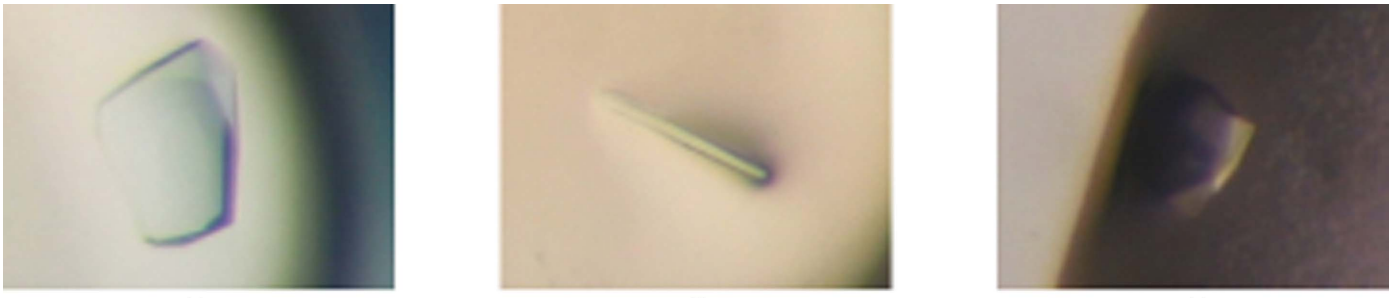

Figure 2
Crystals of SrtC protein were obtained using the sitting-drop method for forms *A* and *B* and the hanging-drop method for form *C*. (*a*) Crystal form *A* (10 × 10 µm) was grown at 293 K with a reservoir solution consisting of 1.6 *M* magnesium sulfate, 0.1 *M* MES pH 6.5. (*b*) Crystal form *B* (20 × 3 µm) was grown at 293 K with a reservoir solution consisting of 0.2 *M* calcium chloride, 0.1 *M* MES pH 6.0. (*c*) Crystal form *C* (20 × 20 µm) was grown at 277 K with a reservoir solution consisting of 0.1 *M* MES pH 7.0, 1.9 *M* ammonium sulfate.

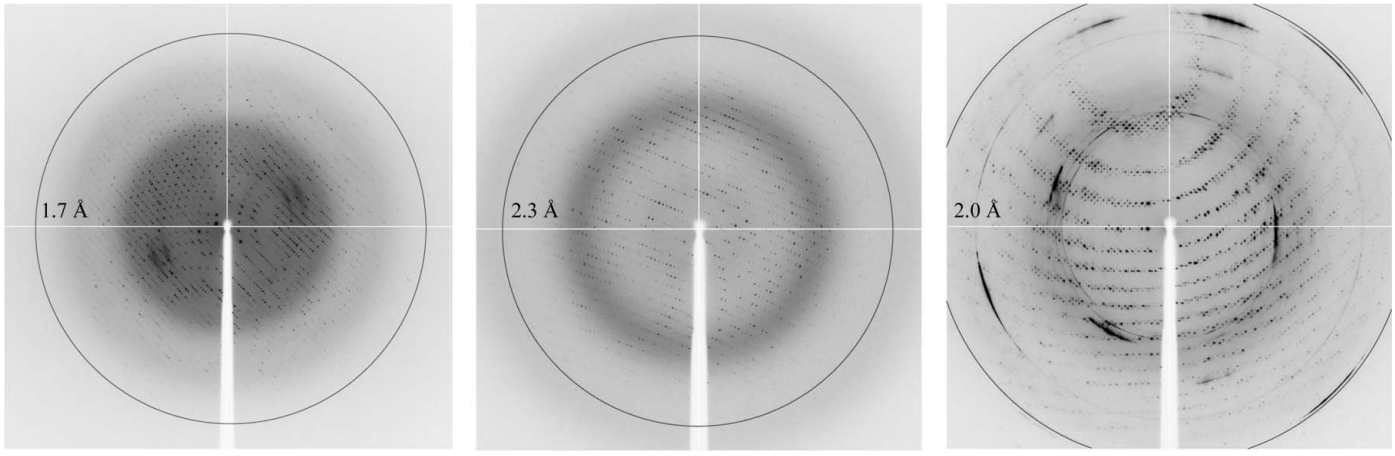

Figure 3
X-ray diffraction patterns recorded from SrtC crystal forms *A* (*a*), *B* (*b*) and *C* (*c*).

Table 1
Data-collection statistics from SrtC crystal forms *A*, *B* and *C*.

Values in parentheses are for the highest resolution shell.

Crystal form	*A*	*B*	*C*
Space group	$P2_12_12_1$	*P*222	*P*222
Unit-cell parameters (Å)	a = 48.9, b = 96.9, c = 98.9	a = 48.8, b = 97.2, c = 99.3	a = 48.6, b = 96.5, c = 98.8
V_M (Å^3 Da^{-1})	2.39	2.45	2.41
Solvent content (%)	49	50	49
No. of molecules in ASU	2	2	2
Resolution range (Å)	42.02–1.70 (1.79–1.70)	49.63–2.30 (2.42–2.30)	49.39–2.00 (2.11–2.00)
Total observed reflections	350859 (46799)	139254 (18974)	228540 (28510)
Unique reflections	52404 (7580)	21707 (3106)	31946 (4490)
Redundancy	6.7 (6.3)	6.4 (6.3)	7.2 (6.3)
Completeness (%)	99.8 (99.2)	100 (100)	99.2 (97.2)
R_{merge}† (%)	9.3 (51.7)	8.8 (28.2)	10.9 (30.5)
$\langle I/\sigma(I)\rangle$	14.6 (3.4)	17.6 (6.3)	16.1 (6.3)

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

crystallization solution were equilibrated against reservoirs containing a larger volume (50 µl) of crystallization solution. Crystallization conditions were also assessed using the hanging-drop vapour-diffusion method in combination with the sparse-matrix screen Crystal Screen 2 (Hampton Research). Diffraction data were collected at the ESRF in Grenoble, France (beamline ID14-1). Data were processed with the program *MOSFLM* (Leslie, 2006) and reduced with *SCALA* (Collaborative Computational Project, Number 4, 1994)

3. Results

The SrtC protein was cloned into plasmid pET24c, expressed in *E. coli* without any tag and purified in five steps (Fig. 1). Production of high yields of a soluble form of SrtC (46–257) required the removal of the peptide signal as well as the hydrophobic area in the C-terminal part that is predicted to be a membrane anchor. Electrospray mass-spectrometric analyses show a mass of 24 013.6 ± 1 Da, which matches the theoretical mass of 24 013.3 Da. As expected from the primary structure, 5,5′-dithiobis-(2-nitrobenzoic acid) titration revealed one reduced cysteine under denaturing and native conditions. This analysis also confirmed the reduced state of the cysteine residue in SrtC.

Three different crystallization conditions have been established for SrtC (Fig. 2). While the first crystal form appeared after 3 d at 293 K in 0.1 *M* MES pH 6.5, 1.8 *M* ammonium sulfate, 0.01 *M* cobalt(II) chloride, two other crystal forms appeared after two months at 293 K in 1.6 *M* magnesium sulfate, 0.1 *M* MES pH 6.5 (crystal form *A*) and 0.2 *M* calcium chloride, 0.1 *M* MES pH 6.0, 20%(*w*/*v*) PEG 6000 (crystal form *B*). Further optimizations were performed using hanging-drop vapour diffusion in order to increase the quality of the crystals that rapidly appeared using reservoir solution containing 0.1 *M* MES pH 7.0 and 1.9 *M* ammonium sulfate (2 µl protein solution at 10 mg ml^{-1} was mixed with 1 µl reservoir solution; Fig. 2). Crystal form *C* appeared after one month of equilibration at 277 K. All crystals were cryoprotected with 20% glycerol; this was followed by flash-cooling in liquid nitrogen.

The three types of crystal were all suitable for diffraction experiments. Crystals forms *A*, *B* and *C* diffracted to 1.7, 2.3 and 2.2 Å resolution, respectively (Fig. 3). The data-collection statistics for crystal forms *A*, *B* and *C* are listed in Table 1. The best data were obtained for crystal *A*. The diffraction pattern indicated that crystal *A* belonged to a primitive orthorhombic crystal system with unit-cell parameters a = 48.91, b = 96.86, c = 98.87 Å, $\alpha = \beta = \gamma = 90^\circ$. Analysis of the Matthews coefficients revealed the possible presence of two monomers in the asymmetric unit of the primitive orthorhombic crystal (Matthews, 1968). Structural determination is in progress using molecular replacement with the reported structure of the homologue SrtA from *Staphylococcus aureus* (30% identity; PDB code 1t2p; Zong *et al.*, 2004) as a search model.

We gratefully acknowledge access to synchrotron radiation at beamline ID14-1 at the ESRF, Grenoble, France as well as the Structural Genomic Consortium Stockholm (SGC), localized within the Department of Medical Biochemistry and Biophysics at the Karolinska Institute, for access to the Phoenix robot. This work was supported by the Swedish Research Council, the Royal Academy of Sciences and Torsten and Ragnar Söderbergs Foundation.

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