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Preliminary crystallographic study of the *Streptococcus agalactiae* sortases, sortase A and sortase C1

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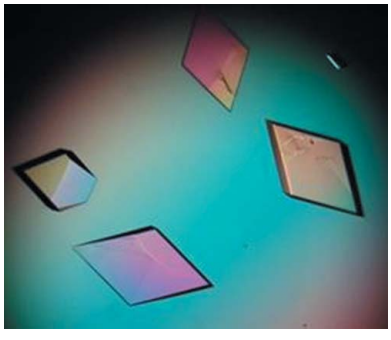


Sortases are cysteine transpeptidases that are essential for the assembly and anchoring of cell-surface adhesins in Gram-positive bacteria. In *Streptococcus agalactiae* (GBS), the pilin-specific sortase SrtC1 catalyzes the polymerization of pilins encoded by pilus island 1 (PI-1) and the housekeeping sortase SrtA is necessary for cell-wall anchoring of the resulting pilus polymers. These sortases are known to utilize different substrates for pilus polymerization and cell-wall anchoring; however, the structural correlates that dictate their substrate specificity have not yet been clearly defined. This report presents the expression, purification and crystallization of SrtC1 (SAG0647) and SrtA (SAG0961) from *S. agalactiae* strain 2603V/R. The GBS SrtC1 has been crystallized in three crystal forms and the GBS SrtA has been crystallized in one crystal form.

1. Introduction

Streptococcus agalactiae, also known as group B *Streptococcus* (GBS), is a major cause of neonatal morbidity and mortality (Dermer *et al.*, 2004). Vertical transmission, resulting from intrauterine infection of the fetus or infection during passage through the birth canal, leads to GBS infections in neonates, which include septicemia, pneumonia and meningitis. Intrapartum prophylaxis has drastically decreased GBS-related neonatal mortality in the past 30 years; however, the pathogen remains a major cause of neonatal disease (Schrag *et al.*, 2000; Arisoy *et al.*, 2003; Larsen & Sever, 2008). GBS is also becoming an increasingly common cause of invasive disease in nonpregnant, elderly or immune-compromised adults. Mortality rates among non-pregnant adults exceed those seen in neonates (Sendi *et al.*, 2008; Edwards *et al.*, 2005; Bolaños *et al.*, 2001; Schuchat, 1998), which has forced the focus of investigations on adult disease. The lack of a universal vaccine against different GBS strains and the emergence of resistance to commonly used antibiotics has necessitated the search for alternative targets for the development of anti-infective drugs (Johri *et al.*, 2006; Heelan *et al.*, 2004; Lin *et al.*, 2000).

Sortase enzymes anchor proteins and protein complexes onto the bacterial cell wall in Gram-positive bacteria and such covalently attached adhesins are involved in a multitude of functions, including host-cell adhesion, nutrient acquisition, host immune evasion *etc*. Of all the sortases, *Staphylococcus aureus* sortase A and sortase B have been extensively investigated (Cossart & Jonquières, 2000; Ton-That & Schneewind, 1999; Ton-That *et al.*, 1999; Mazmanian *et al.*, 1999) and both of them are involved in anchoring surface proteins that perform different functions and carry distinct sorting signals. Sortase enzymes are also known to play a role in the assembly and anchoring of pili in Gram-positive bacteria. Pili are long, thin and filamentous structures attached to the bacterial cell surface which mediate host-tissue adhesion. The presence of pili has been reported in a number of Gram-positive bacteria such as *Corynebacterium diphtheriae*, *Streptococcus pneumoniae* and *S. agalactiae* (Ton-That *et al.*, 2004; Ton-That & Schneewind, 2004; Dramsi *et al.*, 2006). The Gram-positive pili are typically composed of two or more pilin proteins, with the major pilin forming the pilus shaft and the minor pilins present on the tip, the base and distributed along the shaft (Ton-That & Schneewind, 2003; Mandlik *et al.*, 2008; Hilleringmann *et*

al., 2009). Based on their primary structure, pilin-specific sortases differ from housekeeping sortases in their C-terminal transmembrane domain (Bender & Weiser, 2006; Dramsi *et al.*, 2006) and have possible differences in their active-site geometry and composition (Race *et al.*, 2009; Manzano *et al.*, 2008). Nonetheless, the molecular basis that defines the substrate specificity of the two enzyme classes is not yet well understood. Since the pilus composition and organization vary in different bacteria as well as in different strains of the same bacterial species, structural characterization of two classes of sortases that dictate the pili assembly and anchoring from the same bacterial strain is crucial.

Analysis of the eight sequenced genomes of GBS has led to the identification of two genomic islands, each containing genes coding for proteins with LP*X*TG motifs and sortases (Dramsi *et al.*, 2006; Rosini *et al.*, 2006). This is in addition to the presence of an upstream *srtA* locus that encodes the 'housekeeping' sortase A (SrtA) in all analyzed GBS strains (Dramsi *et al.*, 2006). Each island codes for three proteins sufficient for one pilus, and the polymerization of one of the three proteins is essential for the incorporation of the other two proteins into the pilus structure. Two sortases (class C sortases), each having specificity for one of the three components, coded in each island are required to complete the pilus assembly (Rosini *et al.*, 2006; Nobbs *et al.*, 2008). In this report, we present the expression, purification and crystallization of the housekeeping sortase SrtA and the pilin-specific sortase SrtC1 from GBS strain 2603V/R. 2603V/R is a clinical type V isolate which is often associated with invasive infection in nonpregnant adults (Farley, 2001; Davies *et al.*, 2001). SrtC1 of the 2603V/R strain is required for the formation of the PI-1 pilus shaft and SrtA is essential for efficient cell-wall anchoring of the assembled pili. We have expressed and purified a truncated version of GBS SrtC1 comprising residues 43–260 (GBS $SrtC1_{43–260}$) and crystallized the recombinant protein in three different space groups. A truncated version of GBS SrtA (residues 82–238; GBS $SrtA_{82–238}$) has also been crystallized under very different conditions compared with GBS $SrtC1_{43–260}$.

2. Materials and methods

2.1. Sortase A cloning, expression and purification

The primers 5′-TACTTCCAATCCAATGCATCTGCTCAAACGAAATCACATA-3′ and 5′-TTATCCACTTCCAATGTTAAAATGCTTTTAATATCGACTCAT-3′ were used to PCR-amplify the sequence of GBS *srtA* (coding for Ser82–Gln238) from chromosomal DNA of GBS strain 2603V/R (locus tag of SAG0961). The DNA fragment was cloned into pMCSG7 by ligation-independent cloning according to a published protocol (Stols *et al.*, 2002). After verification by DNA sequencing, the resulting plasmid $pSrtA_{S82–Q238}$ was transformed into *Escherichia coli* BL21 (DE3).

GBS $SrtA_{82–238}$ protein was expressed and purified as follows. A litre of Luria–Bertani (LB) broth supplemented with ampicillin (100 µg ml^{-1}) was inoculated at 310 K with a 10 ml overnight culture of *E. coli* BL21 (DE3) harboring $pSrtA_{S82–Q238}$. When the bacterial growth reached an OD_{600} of 0.7–0.8, the temperature was reduced to 303 K and protein expression was induced with 0.5 m*M* isopropyl *β*-D-1-thiogalactopyranoside (IPTG). The bacteria were grown for another 4 h at 303 K and the cells were harvested by centrifugation at 2568*g* for 20 min at 277 K and resuspended in 50 m*M* Tris buffer pH 7.5, 500 m*M* NaCl, 0.1 m*M* phenylmethylsulfonyl fluoride (PMSF), 10%(*v*/*v*) glycerol (lysis buffer). The cell lysate obtained by sonication was centrifuged at 18 000*g* for 30 min at 277 K. The supernatant was loaded onto a column containing 8 ml Ni–NTA Superflow Agarose resin pre-equilibrated with lysis buffer. The column was washed with lysis buffer, followed by washing with an equal volume of 50 m*M* Tris buffer pH 7.5, 100 m*M* NaCl (buffer *A*). The protein was eluted with a linear imidazole gradient (0–300 m*M*) in buffer *A* over 15 column volumes. Fractions containing GBS $SrtA_{82–238}$ were pooled and dialyzed against a buffer containing 50 m*M* Tris pH 7.5, 100 m*M* NaCl, 1 m*M* EDTA, 1 m*M* dithiothreitol (DTT). The His tag was then cleaved by overnight incubation at 277 K with purified recombinant His-tagged TEV protease using a sortase:protease ratio of 100:1. After cleavage of the His tag, the protein retained the N-terminal SNA amino-acid sequence from the TEV cleavage site. The molecular weight of the protein corresponding to this sequence is 17 265.6 Da. The mixture was dialyzed overnight at 277 K against 20 m*M* Tris buffer pH 7.5, 100 m*M* NaCl and applied onto an Ni–NTA column. The flowthrough containing the His-tag-cleaved recombinant GBS $SrtA_{82–238}$ was collected and concentrated for gel-filtration chromatography (HiLoad 26/60 Superdex 75) to eliminate higher molecular-weight contaminants that were still present in the flowthrough; the protein was eluted with 20 m*M* Tris buffer pH 7.2, 100 m*M* NaCl, 5 m*M* *β*-mercaptoethanol. The eluted GBS $SrtA_{82–238}$ was concentrated to 38 mg ml^{-1} as determined by UV absorbance (ε = 7450 M^{-1} cm^{-1} as calculated using the ExPASy Proteomics Server; http://www.expasy.org). The concentrated protein was aliquoted and stored at 193 K.

2.2. Sortase SrtC1 cloning, expression and purification

The primers 5′-AAA**GGATCC**TCTCACGCCAATATTAATGCTT-3′ and 5′-AAA**GGATCC**CTATTGTTGTTGCCTGAAGGTCTT-3′, containing *Bam*HI sites (bold), were used to PCR-amplify the sequence of GBS *srtC1* (coding for Ser43–Gln260) from chromosomal DNA of GBS strain 2603V/R (locus tag of SAG0647). The DNA fragment was digested with *Bam*HI and cloned into pQE30 (Qiagen) precut with *Bam*HI. The resulting plasmid $pSrtC1_{S43–Q260}$ was transformed into *E. coli* XL1Blue prior to verification by mini-induction with IPTG and DNA sequencing.

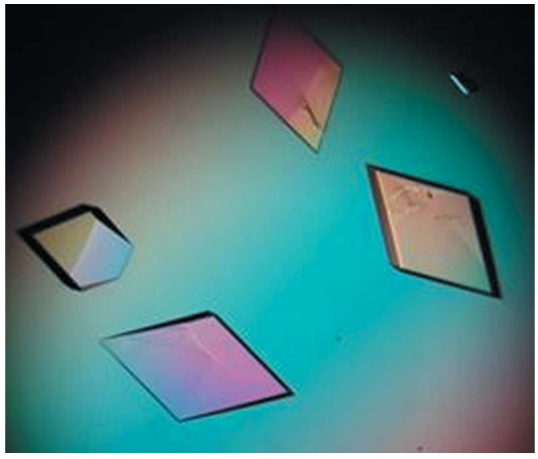
1 l LB medium supplemented with ampicillin (100 µg ml^{-1}) was inoculated with 10 ml culture and the bacteria were grown at 310 K until the optical density at 600 nm reached 0.6–0.8. After induction of protein expression with 1 m*M* IPTG at 303 K, the cells were grown overnight at the same temperature. The cells were harvested by centrifugation at 2568*g* for 15 min at 277 K, resuspended in lysis buffer (20 m*M* sodium phosphate pH 7.4, 150 m*M* NaCl) containing EDTA-free protease-inhibitor cocktail (Roche) and lysed using a sonicator. The cell debris was removed by centrifugation at 48 384*g* for 30 min at 277 K. To purify the protein by affinity chromatography, the supernatant was loaded onto a 5 ml Ni HiTrap chelating column which had been pre-equilibrated with buffer *A* (20 m*M* sodium phosphate pH 7.4, 500 m*M* NaCl). Following elution of the protein with buffer *A* containing 500 m*M* imidazole, the protein was dialyzed overnight against 50 m*M* Tris pH 7.2. GBS $SrtC1_{43–260}$ with a hexahistidine tag (MRGSHHHHHHGS) corresponds to a molecular weight of 25 734.1 Da. The protein obtained from the affinity-chromatography step was fairly pure except for some contaminants which co-eluted with the protein. To separate the contaminants based on their ionic properties (SrtC1 theoretical pI = 7.91 as calculated using the ExPASy Proteomics Server; http://www.expasy.org), GBS $SrtC1_{43–260}$ was loaded onto a Mono S column and eluted with 50 m*M* Tris, 1 *M* NaCl pH 7.2 buffer. The eluted protein was concentrated to 24 mg ml^{-1}, which was determined by monitoring the absorbance at 280 nm (ε = 14 200 M^{-1} cm^{-1} as obtained using *DNAStar* software; DNAStar Inc., Madison, Wisconsin, USA).

2.3. GBS SrtC1$_{43–260}$ crystallization

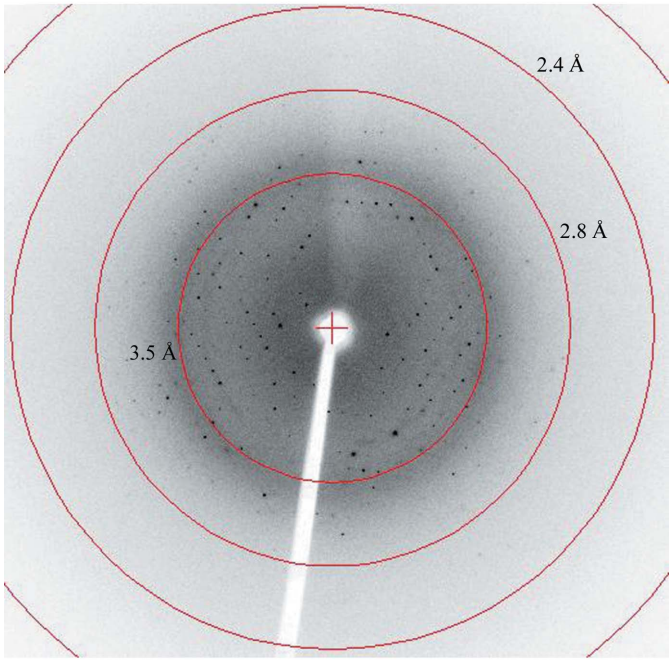
Crystallization trials of GBS SrtC1$_{43–260}$ were carried out using the hanging-drop vapor-diffusion method with the help of a Phoenix Liquid Handling System from Art Robbins Instruments (Sunnyvale, California, USA), which provided a number of leads. Optimizing some of these conditions at 277 K using 1 ml reservoir solution and 2–4 µl hanging drops, with the ratio of protein solution to precipitant well solution remaining constant at 1:1, produced two promising crystal forms. Crystals obtained using 25%(*w*/*v*) PEG 3350, 0.02 *M* ammonium citrate pH 5.2–5.4 at 277 K grew in a week, were sensitive to temperature changes and were also difficult to reproduce. Crystals grown using 15–25%(*w*/*v*) PEG 3350 and 0.2 *M* Tris buffer pH 8.0–8.8 and 0.2 *M* ammonium acetate were reproducible (Fig. 1*a*; type I) and were used for data collection. Further crystallization trials using GBS SrtC1$_{43–260}$ treated with the cysteine protease inhibitors 2-(trimethylammonium)-ethyl-methanethiosulfonate bromide (MTSET) or *n*-[*n*-(L-3-*trans*-carboxyoxirane-2-carbonyl)-l-leucyl]-agmatine (E64) and ammonium sulfate as the precipitant resulted in two crystal forms (types II and III) that were also stable and suitable for data collection. The inhibitors were added to a final concentration of a fivefold excess of the protein concentration used for crystallization and were incubated overnight with SrtC1$_{43–260}$ at 277 K. Rod-shaped (type II) and cube-shaped (type III) crystals, which were both present in the same drop, were obtained for protein incubated with E64 when 2 µl protein solution was mixed with an equal volume of reservoir solution and equilibrated against 1 ml 1.6 *M* ammonium sulfate, 100 m*M* $CaCl_2$ and 100 m*M* sodium cacodylate buffer pH 6.5.

2.4. GBS SrtA$_{82–238}$ crystallization

Preliminary crystallization trials were carried out at 295 K using the hanging-drop vapor-diffusion method with commercially available crystallization screens. The initial drops were made up of 1 µl

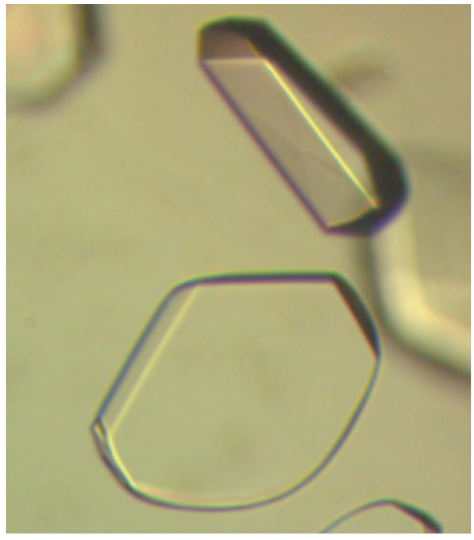


(*a*)

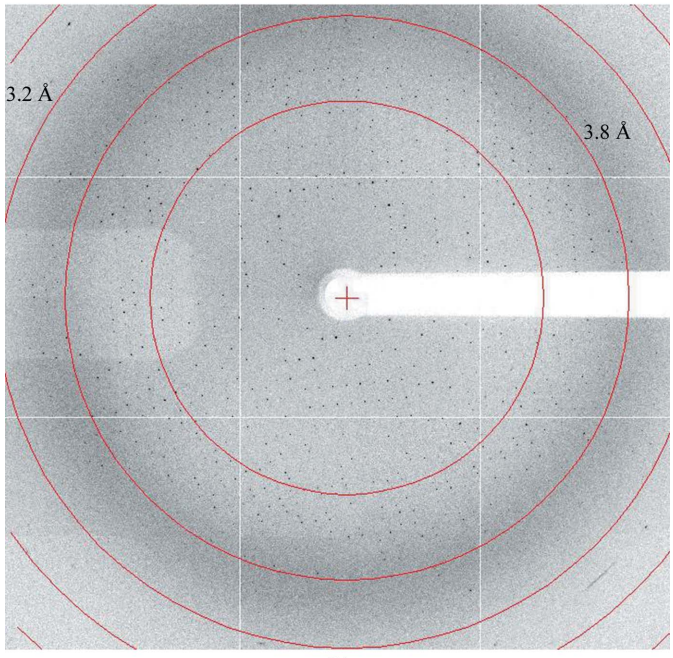


(*b*)

Figure 1
(*a*) Type I crystals of GBS SrtC1$_{43–260}$ belonging to the monoclinic space group *C*2 (0.3 × 0.2 × 0.05 mm). (*b*) X-ray diffraction pattern for the monoclinic type I GBS SrtC1$_{43–260}$ crystals.



(*a*)



(*b*)

Figure 2
(*a*) Crystals of the housekeeping sortase GBS SrtA$_{82–238}$ (0.5 × 0.4 × 0.1 mm). (*b*) X-ray diffraction pattern for the GBS SrtA$_{82–238}$ crystal.

Table 1
Data-collection statistics for GBS SrtC1$_{43–260}$ and GBS SrtA$_{82–238}$ crystals.

Values in parentheses are for the last resolution shell.

	GBS SrtC1$_{43–260}$			
	Type I	Type II	Type III	GBS SrtA$_{82–238}$
No. of crystals	1	1	1	1
X-ray source	In-house	SER-CAT 22-ID	SER-CAT 22-ID	NE-CAT 24-ID
Wavelength (Å)	1.541	1.0	1.0	0.97918
Detector	R-AXIS IV image plate	MAR 300	MAR 300	ADSC 315 CCD
Crystal-to-detector distance (mm)	100	225	200	450
Rotation range per image (°)	1	0.5	0.5	1
Total rotation range (°)	360	360	360	193
Exposure time per image (s)	300	10	2	1
Resolution range (Å)	27.2–2.45 (2.5–2.45)	29.2–2.9 (2.95–2.90)	29.30–3.0 (3.1–3.0)	40–3.1 (3.2–3.10)
Space group	*C*2	$P2_12_12_1$	$P3_121$	*C*2
Unit-cell parameters				
a (Å)	78.6	69.1	70.9	237.7
b (Å)	46.9	73.4	70.9	167.7
c (Å)	66.6	127.3	195.7	97.5
β (°)	115.0		120.0	93.5
Mosaicity (°)	0.3	0.3	0.6	0.37
Total No. of measured intensities	59427 (5927)	110877 (5375)	125451 (12302)	285320 (28495)
Unique reflections	8218 (811)	15115 (721)	22041 (2241)	68853 (6819)
Multiplicity	7.2 (7.0)	7.3 (7.3)	5.7 (5.6)	4.2 (4.3)
Mean $I/\sigma(I)$	33.5 (13.1)	57.5 (20.3)	11.3 (4.8)	16.4 (1.85)
Completeness (%)	100 (100)	100 (100)	99.9 (100)	99.7 (99.6)
χ^2	0.98 (0.94)	2.76 (1.28)	0.98 (1.1)	1.66 (1.41)
R_{merge}† (%)	4.0 (12.5)	5.0 (12.1)	8.8 (24.8)	0.11 (84.1)
Overall *B* factor from Wilson plot (Å^2)	51.1	62.7	95.2	79.7
No. of molecules in the asymmetric unit	1	2	2	18

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ are the intensities of symmetry-related reflections and $\langle I(hkl)\rangle$ is the average intensity over all observations.

protein solution (19 mg ml^{-1} in 20 m*M* Tris buffer pH 7.2, 100 m*M* NaCl, 5 m*M* *β*-mercaptoethanol) and 1 µl reservoir solution. Drops were equilibrated against 500 µl reservoir solution consisting of 4–8%(*w*/*v*) PEG 8000, 0.1–0.2 *M* imidazole pH 6.5–6.8 and 0.2–0.6 *M* zinc acetate. Further optimization by replacing zinc acetate with zinc sulfate produced suitable crystals in one week (Fig. 2*a*).

2.5. Data collection and processing

GBS SrtC1$_{43–260}$ crystals were cryoprotected using 20%(*v*/*v*) ethylene glycol mixed with stabilizing reservoir solution. Native diffraction data from type I crystals were collected using an R-AXIS IV detector on an in-house Rigaku rotating-anode X-ray generator operating at 100 mA. Diffraction data from type II and type III crystals were collected on the SER-CAT 22-ID beamline at the Advanced Photon Source (APS), Argonne National Laboratory (ANL), Chicago (Fig. 1*b*). The type I, II and III GBS SrtC1$_{43–260}$ crystals diffracted to 2.45, 2.9 and 3.0 Å resolution and belonged to space groups *C*2, $P2_12_12_1$ and $P3_121$, respectively (Table 1). Diffraction data sets were processed and scaled using the *d*TREK* (Pflugrath, 1999) or *HKL*-2000 (Otwinowski *et al.*, 2003) programs.

Crystals of GBS SrtA$_{82–238}$ were cryoprotected by a quick soak in a stabilizing solution consisting of reservoir solution containing 20–25%(*v*/*v*) glycerol. The crystals belonged to space group *C*2 and diffracted to 2.9 Å resolution on the NE-CAT 24-ID beamline at APS (Fig. 2*b*). Diffraction data were collected at 100 K and were processed and scaled using *HKL*-2000 (Otwinowski *et al.*, 2003).

3. Results and discussion

Assuming the presence of one molecule in the asymmetric unit, the calculated Matthews coefficient V_M (Matthews, 1968) is 2.57 Å^3 Da^{-1} for type I GBS SrtC1$_{43–260}$ crystals belonging to the monoclinic space group *C*2 and the estimated solvent content is 52%. Assuming the presence of two molecules in the asymmetric unit, the type II and III crystals of GBS SrtC1$_{43–260}$, which belonged to the orthorhombic $P2_12_12_1$ and trigonal $P3_121$ space groups, have comparable solvent contents (55–60%), with calculated V_M values (Matthews, 1968) of 2.76 and 3.13 Å^3 Da^{-1}, respectively.

GBS SrtC1$_{43–260}$ shares less than 30% sequence identity with the two *Staphylococcus aureus* sortases Srt A and Srt B and has 56 and 51% identity to *Streptococcus pyogenes* sortases C1 and C2, respectively. Attempts to solve the crystal structure of GBS SrtC1$_{43–260}$ by molecular-replacement methods using other known crystal structures were not successful. Since the GBS SrtC1$_{43–260}$ sequence contains two methionine residues, attempts are under way to generate an SeMet derivative of recombinant GBS SrtC1$_{43–260}$ that is suitable for MAD/SAD phasing.

The GBS SrtA$_{82–238}$ crystals belonged to space group *C*2. Their calculated solvent content was 58% and the Matthews coefficient (Matthews, 1968) was 2.94 Å^3 Da^{-1} when 18 molecules were assumed to be present in the asymmetric unit. Attempts to solve the crystal structure of GBS SrtA$_{82–238}$ by molecular-replacement methods were unsuccessful. Generation of an SeMet derivative of GBS SrtA$_{82–238}$ is in progress and we will attempt to determine the crystal structure by MAD/SAD phasing. In addition, we are continuing in our efforts to generate different crystal forms of GBS SrtA$_{82–238}$, possibly with a lower number of molecules in the asymmetric unit.

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