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Expression, purification, crystallization and preliminary X-ray diffraction studies of the human keratin 4-binding domain of serine-rich repeat protein 1 from *Streptococcus agalactiae*

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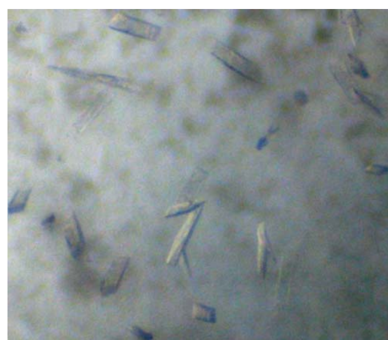
Serine-rich repeat protein 1 (Srr-1) is a surface protein from *Streptococcus agalactiae*. A 17 kDa region of this protein has been identified to bind to human keratin 4 (K4) and is termed the Srr-1 K4-binding domain (Srr-1-K4BD). Recombinant Srr-1-K4BD was overexpressed in *Escherichia coli* BL21 (DE3) cells. Native and selenomethionine-substituted proteins were prepared using Luria–Bertani (LB) and M9 minimal media, respectively. A two-step purification protocol was carried out to obtain a final homogenous sample of Srr-1-K4BD. Crystals of native Srr-1-K4BD were obtained using PEG 3350 as a precipitant. The crystals diffracted to 3.8 Å resolution using synchrotron radiation and belonged to space group $P2_1$, with unit-cell parameters a = 47.56, b = 59.48, c = 94.71 Å, β = 93.95°.



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1. Introduction

Streptococcus agalactiae, also called group B streptococcus (GBS), is a Gram-positive streptococcal bacterium and a causative agent of invasive neonatal sepsis and meningitis (Glaser *et al.*, 2002). Immunocompromised adults who acquire invasive GBS infections may develop infections of the bloodstream (sepsis), lung (pneumonia) or, rarely, the fluid and lining tissues surrounding the brain (meningitis). GBS is observed in 15–45% of all healthy women as a commensal bacterium in their intestine, vagina and rectum (Gilbert, 2004).

Both commensal and pathogenic lifestyles necessitate the attachment of bacteria to host tissues (Doran & Nizet, 2004; Jenkinson & Lamont, 1997; Patti *et al.*, 1994). Surface proteins are known to mediate this key step of bacteria–host interaction in many microbial infections (Navarre & Schneewind, 1999). *S. agalactiae* expresses many surface-exposed proteins that are known to adhere to the extracellular matrix (ECM) molecules of the host. For example, the proteins FbsA and FbsB bind to fibrinogen (Schubert *et al.*, 2002), Lmb binds to laminin (Spellerberg *et al.*, 1999) and ScpB (C5a peptidase) attaches to fibronectin (Beckmann *et al.*, 2002; Cheng *et al.*, 2002).

Serine-rich repeat protein 1, termed Srr-1 (1310 amino acids), is one such surface protein encoded by the *gbs1529* gene of *S. agalactiae* (Seifert *et al.*, 2006). Srr-1 (UniProt accession Q8E473) belongs to the streptococcal and staphylococcal serine-rich repeat (Srr) protein family. The Srr family of proteins contain a short serine-rich repeat region in the N-terminal region, a nonrepeat adhesive region, a region containing numerous serine-rich repeats, and a cell-wall-anchoring region comprising an LP*X*TG motif, a hydrophobic domain and a short C-terminal charged tail (Fig. 1; Navarre & Schneewind, 1999; Samen *et al.*, 2007). A previous study identified a

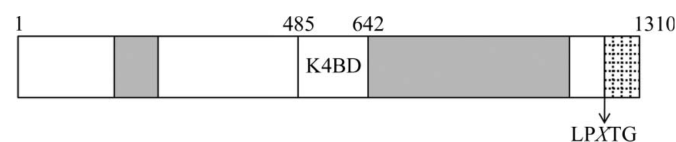

Figure 1
Structural organization of *S. agalactiae* Srr-1. Srr-1 contains 1310 amino acids. The human keratin 4-binding domain (K4BD) is shown. The serine-rich repeat regions are indicated by grey boxes. At the C-terminal end the LP*X*TG motif required for sortase-mediated anchoring of the protein to the cell-wall peptidoglycan and the membrane-spanning region (dotted box) are indicated.

17 kDa region (residues 485–642) of Srr-1 as a human keratin 4 binding domain (Samen *et al.*, 2007) termed Srr-1-K4BD. In that study, five different constructs, namely Srr-1-N (residues 7–642), Srr-1-N1 (7–327), Srr-1-N2 (322–485), Srr-1-N3 (485–642) and Srr-1-N2N3 (322–642), were produced, and using binding assays it was demonstrated that Srr-1-N3 (Srr-1-K4BD) is sufficient and necessary for binding to human keratin 4.

Srr-1 is present in many GBS strains and a previous study showed that Srr-1 promotes penetration of the blood–brain barrier (BBB), causing invasion of the central nervous system (CNS) and leading to the development of GBS meningitis (van Sorge *et al.*, 2009). Despite universal screening and preventive measures, early-onset GBS disease still prevails and remains a significant public health issue. Maternal immunization against GBS is an excellent way to prevent not only neonatal disease but also stillbirth and maternal disease. Vaccines against GBS are expected to be the most effective and sustainable long-term preventive strategy (Gilbert, 2004). Since the adhesin Srr-1 paves the way for the critical step in microbial infection, which is the attachment of the bacterium to the host molecule, understanding the structure–function relationship of Srr-1-K4BD will aid in the development of new therapeutics for GBS infection.

Here, we report the expression, purification, crystallization and preliminary X-ray analysis of recombinant Srr-1-K4BD.

2. Materials and methods

2.1. Expression and purification of Srr-1-K4BD

The *srr-1* gene encoding the region comprised of residues 485–642 (Srr-1-K4BD) was PCR-amplified using the primers CATG**CCATGG**TCATGAAGCTTGATGATGAAAGAC and CCGC**GGATCC**TCTGATAAAAGTTTAATTTCGGC and cloned into the pET-28a(+) expression vector (Novagen) using the restriction enzymes *Nco*I and *Bam*HI (sites shown in bold) as reported previously (Samen *et al.*, 2007). The pET-28a::*Srr-1-K4BD* construct was transformed into *Escherichia coli* BL21 (DE3) cells and protein expression was carried out in Luria–Bertani (LB) medium. A 10 ml overnight culture was prepared and transferred to 1 l LB medium supplemented with 50 µg ml^{-1} kanamycin and allowed to grow at 310 K until an optimum optical density (OD_{600} of 0.6–0.8) was reached. Following this, protein expression was induced with 1 m*M* isopropyl β-D-1-thiogalactopyranoside (IPTG). After 4 h induction, the cells were spun down at 2468*g* for 20 min at 277 K. The harvested cells were resuspended in 15 ml buffer *A* (20 m*M* Tris–HCl pH 7.0, 300 m*M* NaCl, 5% glycerol, 5 m*M* β-mercaptoethanol) with 1 m*M* phenylmethylsulfonyl fluoride (PMSF). The cell suspension was lysed by ultrasonication on ice. The lysate was spun down at 9838*g* for 40 min. The supernatant and pellet were checked on a 15% SDS–PAGE gel and the protein was found in the soluble fraction (Fig. 2).

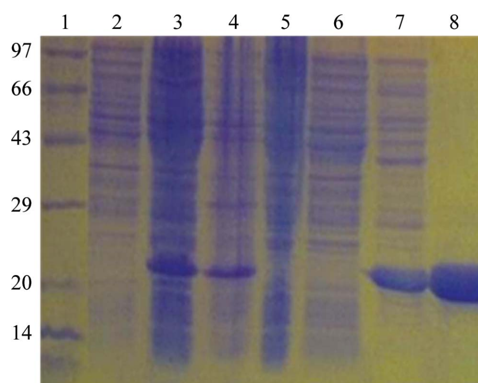

Figure 2
Expression and purification analysis of recombinant Srr-1-K4BD on a 15% SDS–PAGE gel. Lane 1, molecular-weight marker (labelled in kDa); lane 2, cell lysate before IPTG induction; lane 3, supernatant after lysis; lane 4, pellet after lysis; lane 5, unbound material from Ni–NTA column; lane 6, buffer wash with 10 ml buffer *A*; lane 7, pooled fractions of protein eluted from Ni–NTA column with 100–250 m*M* imidazole; lane 8, final purified sample after size-exclusion chromatography.

Initial purification was carried out by the immobilized metal-affinity chromatography (IMAC) technique using an Ni–NTA column. The bound protein was eluted using a linear gradient of 0–1 *M* imidazole in buffer *A*. The peak fractions corresponding to Srr-1-K4BD were collected and checked on a 15% SDS–PAGE gel (Fig. 2). The fractions containing the protein were pooled and concentrated to 2 ml using a Vivaspin 20 concentrator (Sartorius Stedim Biotech). The yield of the protein was around 9 mg per litre of culture.

The concentrated sample was further purified using size-exclusion chromatography by loading it onto a Superdex 75 column (130 ml; GE Healthcare) pre-equilibrated with a buffer consisting of 20 m*M* Tris–HCl pH 7.0, 100 m*M* NaCl, 5% glycerol, 5 m*M* β-mercaptoethanol. The fractions corresponding to the peak were subjected to 15% SDS–PAGE and pure fractions were pooled and concentrated to 200 µl. A final yield of 5.6 mg protein was obtained from 1 l culture and was concentrated to 28 mg ml^{-1} for crystallization trials.

Selenomethionine incorporation of Srr-1-K4BD was carried out using the methionine-auxotroph strain *E. coli* B834 (DE3) (Novagen). A 10 ml overnight culture was grown in LB medium at 310 K. The cells were harvested at 2468*g* for 20 min and the cell pellet was used as an inoculum for 1 l 1× M9 minimal medium supplemented with kanamycin (50 µg ml^{-1}) and other additives (Sambrook *et al.*, 1989; Guerrero *et al.*, 2001). L-Selenomethionine (40 µg ml^{-1}) was added 1 h after inoculation. The culture was grown for 10 h before IPTG induction. A cell-harvesting and purification protocol similar to that used for native Srr-1-K4BD was carried out except that the buffers were degassed in order to avoid oxidation of selenomethionine.

2.2. Crystallization

Several trials were conducted to crystallize the Srr-1-K4BD protein. Initial conditions were obtained using commercial screens (Hampton Research) and home-made screens of ammonium sulfate *versus* pH (4–9) and polyethylene glycol (PEG; 600, 800, 1500 and 3500) *versus* pH (4–9). Crystallization was performed using the hanging-drop vapour-diffusion method at 293 K. 1 µl protein sample was mixed with an equal volume of reservoir solution and equilibrated against 1 ml of the latter at 293 K. Small two-dimensional plate-like crystals were obtained in 2 d in conditions with 32–35%(*w*/*v*) PEG 3350 as a precipitant and imidazole (pH 6.3 and 6.5) as a buffer. To obtain diffraction-quality crystals, extensive trials such as reducing the volume of the reservoir solution (from 1000 to 600 µl), changing the amounts of protein solution and reservoir solution in the drop (1 + 1 µl, 2 + 2 µl, 2 + 3 µl and 3 + 2 µl), addition of reducing agents and salts to the reservoir solution and microseeding (Bergfors, 2009) were carried out.

A microseed stock solution was prepared by adding 10 µl mother liquor to a crystallization drop which contained very thin plate-like crystals. This was pipetted into an 1.5 ml Eppendorf tube kept on ice. 50–100 µl mother liquor was added and spun down to wash the crystals. The supernatant was discarded and 50–100 µl mother liquor was again added and spun down. This procedure was repeated three times to wash the crystals. 50 µl mother liquor was then added to the washed crystals and a small glass bead was placed in the Eppendorf tube. The tube was vortexed for a few minutes to crush the crystals.

This 50 µl solution was used as a seed stock. From this, dilutions of 1:50, 1:100 *etc.* were made which were used to streak the crystallization drops. Finally, a microseeded drop (1:100 dilution, streaked using a whisker) containing 1 µl protein solution and 1 µl reservoir solution [35%(*w*/*v*) PEG 3350, 0.1 *M* imidazole pH 6.5, 40 m*M* $MgCl_2$] equilibrated against 1 ml reservoir solution gave stable native crystals of Srr-1-K4BD (0.3 × 0.15 × 0.05 mm) in one week (Fig. 3).

2.3. Data collection and processing

Initial diffraction experiments were carried out using an in-house MAR 345 image-plate detector and Bruker MICROSTAR copper rotating-anode generator operating at 60 mA and 45 kV. Crystals were taken directly from the crystallization drop and flash-cooled in a liquid-nitrogen stream. The PEG 3350 in the crystallization drop acted as a cryoprotectant. The crystals diffracted to 5.0 Å resolution with an exposure time of 5 min per frame. Subsequently, diffraction experiments were carried out at the Elettra synchrotron-radiation laboratory in Italy. A native data set was collected on the XRD1 beamline from one crystal at 100 K. An oscillation range of 1° was used to collect 271 frames with an exposure of 55 s per frame at a crystal-to-detector distance of 150 mm. The diffraction data were processed using the program *iMOSFLM* (Battye *et al.*, 2011). Data-collection statistics are given in Table 1.

3. Results and discussion

Assuming a molecular weight of 17 kDa for the protein and three molecules in the asymmetric unit, the resultant Matthews coefficient (V_M; Matthews, 1968) was calculated to be 2.62 $Å^3\ Da^{-1}$ with a solvent content of 53.06%. Alternatively, if there were two molecules in the asymmetric unit the Matthews coefficient was calculated to be 3.93 $Å^3\ Da^{-1}$ with a solvent content of 68.71%. To determine the location of the noncrystallographic twofold or threefold symmetry, a self-rotation function study was performed with the program *POLARRFN* (Winn *et al.*, 2011) using data between 20 and 4 Å resolution. The results of this study were inconclusive and therefore the noncrystallographic symmetry will be determined during subsequent structure-solution steps.

Srr-1-K4BD does not have a homologous structure in the PDB and therefore determination of its structure by molecular replacement is not possible. Srr-1-K4BD contains three methionine residues which can be modified to selenomethionine (SeMet) in order to resolve the phase problem using the multi-wavelength anomalous dispersion (MAD) or single-wavelength anomalous dispersion (SAD) techniques. SeMet-substituted protein was prepared using a methionine-auxotroph strain in minimal medium (M9 medium) as described in §2. Microcrystals of SeMet-substituted Srr-1-K4BD were obtained under similar conditions to those used for the native protein. However, the crystals were too small (the longest dimension was about 0.05 mm) to mount in a loop for diffraction analysis. Further crystallization trials are under way in order to obtain diffraction-quality crystals of SeMet-incorporated Srr-1-K4BD. A three-wavelength MAD data set or a SAD data set will be collected to solve the structure of Srr-1-K4BD.

Figure 3
Thin plate-like crystals of Srr-1-K4BD. The scale bar represents 0.1 mm.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	0.9789
Space group	$P2_1$
Unit-cell parameters	
a (Å)	47.56
b (Å)	59.48
c (Å)	94.71
β (°)	93.95
Resolution (Å)	28.44–3.80 (4.01–3.80)
Total No. of reflections	23741
No. of unique reflections	5320
Completeness (%)	99.5 (100)
R_{merge}† (%)	11.8 (39.7)
$\langle I/\sigma(I)\rangle$	8.3 (3.6)
Average mosaicity (°)	1.5
Multiplicity	4.1

† $R_{merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean of the intensity measurements $I_i(hkl)$ and the summation extends over all reflections.

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