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Cloning, expression, crystallization and preliminary X-ray diffraction studies of staphylococcal superantigen-like protein 1 (SSL1)

Staphylococcus aureus produces a family of exotoxins which are structural homologues of superantigens and thus are called staphylococcal superantigen-like proteins (SSLs). Amongst the 14 SSL genes, *ssl1* (SAOUHSC_00383) has been cloned in the pQE30 expression vector, overexpressed in *Escherichia coli* M15 (pREP4) cells and the protein purified to homogeneity. The protein was crystallized using 6% Tacsimate pH 6.0, 0.1 M MES pH 6.0, 25%(w/v) polyethylene glycol 3350, 100 mM NDSB 256 at 298 K by the sitting-drop vapour-diffusion method. The crystals belonged to space group $P2_1$, with unit-cell parameters $a = 77.9$, $b = 70.5$, $c = 126.5$ Å, $\beta = 106.2^\circ$. X-ray diffraction data were collected and processed to a maximum resolution of 2.5 Å. The crystal contains six molecules in the asymmetric unit.

1. Introduction

Staphylococcus aureus is an opportunistic human pathogen that causes severe diseases such as toxic shock syndrome (TSS), endocarditis and purpura fulminans (Dinges *et al.*, 2000; Nair *et al.*, 2000; Kravitz *et al.*, 2005). Pathogens invading the host are eradicated by innate and adaptive immune responses. *S. aureus* retaliates against host immune defences by producing an array of proteins that are expressed on the cell surface or secreted and interact with the immune-response machinery of the host. Many of these genes are clustered within three pathogenicity islands called staphylococcal pathogenicity island 1 (SaPin1), SaPin2 and SaPin3 (Kuroda *et al.*, 2001; Novick, 2003). SaPin1 and SaPin3 encode an important class of secretory proteins called superantigens (Sags). Sags, which consist of a typical N-terminal β -barrel domain and a C-terminal β -grasp motif (Fraser & Proft, 2008), bind to the outer surface of both the major histocompatibility complex (MHC) class II and the V β region of the T-cell receptors (TCRs; Arcus *et al.*, 2000). Sags nonspecifically activate a large number of T cells, causing the sudden release of a large amount of cytokine, leading to toxic shock syndrome (Fraser & Proft, 2008). Other than Sags, there are 14 superantigen-like proteins (SSLs) which are structural homologues of Sags. SaPin2 consists of 14 SSLs. 11 of these 14 SSLs are arranged in an almost contiguous region and SSL12, SSL13 and SSL14 are clustered somewhat downstream from this region (Arcus *et al.*, 2002; Fraser & Proft, 2008). SSLs display structural similarity to Sags, but do not possess superantigenic activity. SSLs target several key elements of the host innate immune system (Fraser & Proft, 2008; Kotb, 1995; Marrack & Kappler, 1990).

SSL proteins are involved in immune evasion by interacting specifically with opsonins, the complement system and phagocytes which are components of the innate immune system. Among the few structurally and functionally characterized SSLs, SSL7 has been the most studied and has been found to interact with monomeric and secretory forms of immunoglobulin A (IgA). The Fc α RI (CD89) receptor of phagocytes recognizes the Fc region of IgA for opsonization. SSL7 also targets the Fc region of IgA. The binding of SSL7 to IgA prevents the binding of IgA to Fc α RI, resulting in the inhibition of opsonization (Langley *et al.*, 2005). Hence, the binding of SSL7 to IgA facilitates bacterial survival at the mucosal surface, preventing the activation of phagocytes and the complement system. In addition,

the C-terminal β -grasp motif of SSL7 binds to the complement factor C5 (Langley *et al.*, 2005) and inhibits complement-mediated lysis of *S. aureus*. Like SSL7, binding of SSL10 to IgG inhibits its interaction with complement C1q and the Fc γ receptor (Itoh *et al.*, 2013; Patel *et al.*, 2010). SSL5 and its closest sequence homologue SSL11 are found to inhibit the recruitment of phagocytes into the inflammatory and haemorrhagic area, impairing the first line of defence against invading pathogens. SSL5 and SSL11 specifically bind P-selectin glycoprotein ligand 1 (PSGL-1) expressed on the leukocytes, which is a major player in the phagocytosis-mediated immune response (Bestebroer *et al.*, 2007; Chung *et al.*, 2007). SSL5 inhibits the binding of PSGL-1 to P-selectin, an adhesion molecule expressed on activated endothelial cells, and prevents neutrophil extravasations. SSL3 and SSL4 have been found to inhibit Toll-like receptor 2 (TLR2) expressed on the surface of dendritic cells, macrophages and neutrophils. The production levels of certain cytokines and chemokines are suppressed by inhibition of TLR2 activation (Bardoel *et al.*, 2012; Yokoyama *et al.*, 2012). Thus, the structure of the complex of SSL and its interaction partner reveals the mechanism of their interaction, thus delineating some of the immune-evasion strategies of *S. aureus*. The carbohydrate-binding residues have been identified from the crystal structure of SSL5 complexed with sialyl Lewis X tetrasaccharide (sLeX), a glycan chain of PSGL-1 glycoprotein. The carbohydrate-binding residues of SSL5 are conserved among several other SSLs (Baker *et al.*, 2007). The structure of the SSL7-C5-cobra venom factor (CVF) complex established the mechanism of C5 inhibition by SSL7 (Laursen *et al.*, 2011). The inhibition is enhanced when SSL7 binds to both C5 and IgA. CVF is a homologue of the C3b subunit of C3 convertase, which is involved in the activation of C5. Binding of CVF to C5 leads to conformational changes in C5, which guide the cleavage and activation of C5 by a serine protease. It is evident from the structures of the C5-SSL7 and IgA-SSL7 complexes that the simultaneous binding of SSL7 to C5 and IgA interferes with the binding of C5 to convertases (Laursen *et al.*, 2010).

However, among the 14 homologous SSL genes, the structural and functional properties of only a few genes have been determined. Here, we report the crystallization conditions and preliminary data analysis of SSL1, which will provide structural information that may be correlated with known structures of different Sags, SSLs and toxins to shed light on the possible function of SSL1 in host immune evasion.

Figure 1
Crystals of SSL1 as grown using 6% Tacsimate pH 6.0, 0.1 M MES pH 6.0, 25% PEG 3350, 100 mM NDSB 256.

2. Materials and methods

2.1. Cloning

The sequence corresponding to the open reading frame of SSL1 with the N-terminal 25 residues truncated (SAOUHSC_00383) was amplified by polymerase chain reaction (PCR) using genomic DNA of *S. aureus* NCTC 8325 as template. This truncated construct, SSL1^{26–226}, has been designed to remove the predicted signal peptide sequence (SignalP 4.1 server; Petersen *et al.*, 2011) using primers 5'-CGGGATCCCAATCAGTACAAGC-3' (forward primer with *Bam*HI recognition site shown in bold) and 5'-CCCAAGCTT-TTATTTCTATTCTACTAGAATTTTA-3' (reverse primer with *Hind*III recognition site shown in bold). The amplified PCR product was purified and cloned into *Bam*HI- and *Hind*III-digested pQE30 expression vector (Qiagen, USA). Six repeated histidine codons are present upstream of the multiple cloning sites (MCS) of the pQE30 vector. Therefore, the construct for the recombinant protein contains six consecutive histidine codons followed by the codons for glycine and serine (for the *Bam*HI site) as extra residues. The recombinant plasmid DNA was transformed into chemically competent *Escherichia coli* M15 (pREP4) cells and subsequently selected on ampicillin/kanamycin plates. The positive clones were verified by DNA sequencing.

2.2. Overexpression and purification

A positive clone containing the desired construct SSL1^{26–226} was grown in Luria-Bertani broth supplemented with ampicillin (100 μ g ml⁻¹) and kanamycin (25 μ g ml⁻¹) at 310 K until the OD₆₀₀ reached 0.6, followed by induction with 100 μ M IPTG. Cells were grown for a further 4 h at the same temperature to maximize the protein yield. The harvested cells from 1 l culture were resuspended in buffer A (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 10 mM imidazole) containing leupeptin, pepstatin and aprotinin (0.1 μ M each) and 0.02 mM phenylmethylsulfonyl fluoride (PMSF). The cells were then lysed by ultrasonication and the lysate was centrifuged at 22 000g for 40 min. The supernatant was loaded onto Ni-Sepharose High Performance Affinity Matrix (GE Healthcare Biosciences) pre-equilibrated with buffer A. The column was then washed extensively with buffer A followed by buffer B (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 50 mM imidazole) for the removal of nonspecifically bound contaminants. The protein was eluted with buffer C (10 mM Tris-HCl pH 8.0, 300 mM NaCl, 100 mM imidazole). The eluted protein was subjected to size-exclusion chromatography using a Superdex 75 prep-grade matrix in a 16/70C column (GE Healthcare Biosciences) equilibrated with buffer D (10 mM Tris-HCl pH 8.0, 50 mM NaCl) on an ÄKTAprime Plus system (GE Healthcare Biosciences). Fractions of 2 ml were collected at a flow rate of 1 ml min⁻¹. The fractions containing the desired protein were pooled together. The protein concentration was estimated by the method of Bradford (1976) and the purity was verified by 12% SDS-PAGE.

2.3. Crystallization

The purified protein was concentrated to 14.6 mg ml⁻¹ in buffer D using a 10 kDa cutoff Vivaspin 20 concentrator (GE Healthcare). Preliminary screening for crystallization conditions was performed by the sitting-drop vapour-diffusion method using Crystal Screen, Crystal Screen 2, Index, PEGRx 1 and PEGRx 2 (Hampton Research, USA) at 298 K by mixing 2 μ l droplets of concentrated protein solution with equal volumes of reservoir solution in a 96-well sitting-drop Corning plate (Corning Inc., New York, USA) equilibrated against 100 μ l of reservoir solution. A total of 288 conditions

Table 1

Data-collection and processing statistics.

Values in parentheses are for the highest resolution shell.

Wavelength (Å)	1.5418
Space group	$P2_1$
Unit-cell parameters (Å, °)	$a = 77.9, b = 70.5, c = 126.5,$ $\beta = 106.2$
Matthews coefficient V_M (Å ³ Da ⁻¹)	2.42
Solvent content (%)	49.15
Monomer per asymmetric unit	6
Resolution range (Å)	19.68–2.5 (2.63–2.5)
Total No. of observations	197173 (28252)
No. of unique reflections	45615 (6561)
Multiplicity	4.3 (4.3)
Completeness (%)	99.4 (98.7)
Average $I/\sigma(I)$	20.3 (3.9)
R_{int} (%)	6.9 (44.9)
R_{merge} (%)	6.1 (39.3)
$CC_{1/2}$	99.9 (90.8)

† $R_{\text{int}} = \sum_{hkl} \{N(hkl) / [N(hkl) - 1]\}^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of the i th reflection of reflection hkl . ‡ $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of the i th reflection and $\langle I(hkl) \rangle$ is the mean value for all equivalent measurements of reflection hkl . § $CC_{1/2}$ is the extent of internal consistency of the correlation coefficient (CC) between two data subsets which is generated by a random half of the measurements of each unique reflection (Karplus & Diederichs, 2012).

were screened manually to obtain the crystallization conditions. Broom-shaped crystals were obtained from the conditions (i) 0.1 M sodium chloride, 0.1 M HEPES pH 7.5, 1.6 M ammonium sulfate, (ii) 0.1 M HEPES pH 7.5, 20% (w/v) polyethylene glycol (PEG) 10 000 and plate-like crystals were obtained from the condition 6% Tacsimate pH 6.0 (Tacsimate is a trademark of Hampton Research, USA), 0.1 M MES pH 6.0, 25% PEG 4000. The plate-like crystals were further optimized using various concentrations of Tacsimate *versus* PEG of different molecular weights. Crystals with poor diffraction quality were obtained from the condition 6% Tacsimate pH 6.0, 0.1 M MES pH 6.0, 25% PEG 3350. Hence, an additive screen (Hampton Research, USA) was performed and the presence of 100 mM NDSB 256 (nondetergent sulfbetaine, molecular weight 257.37; Hampton

Research, USA) as an additive improved the crystal quality (Fig. 1), enhancing the resolution of diffraction to 2.5 Å.

2.4. Data collection and processing

Before data collection, the crystals were quick-soaked in a cryo-protectant solution consisting of 10% (v/v) glycerol, 6% Tacsimate pH 6.0, 0.1 M MES pH 6.0, 25% PEG 3350 and flash-cooled in a nitrogen stream at 100 K. X-ray diffraction data were collected on an in-house Rigaku R-Axis IV⁺⁺ image-plate detector using Cu $K\alpha$ X-rays generated by a Rigaku MicroMax-007 HF rotating-anode generator. The crystal-to-detector distance was maintained at 170 mm and the crystal was rotated through a total of 206° with 0.5° rotation and an exposure time of 3 min per frame. The crystal diffracted to a resolution of 2.5 Å. The 412 images were processed with *XDS* (Kabsch, 2010) in space group $P2_1$ and the resulting R_{merge} was 6.1%. The space group was determined to be $P2_1$ by observing the systematic absences and was confirmed with *POINTLESS* (Evans, 2006) from the *CCP4* suite (Winn *et al.*, 2011). Scaling was performed using *SCALA* (Evans, 2006) from the *CCP4* suite. The final statistics of data collection and processing are tabulated in Table 1.

3. Results and discussion

Superantigen-like protein 1 (SSL1^{26–226}) from *S. aureus* was successfully cloned in *E. coli* and purified to homogeneity with an N-terminal His₆ tag. The molecular weight of monomeric His₆ SSL1^{26–226} (23 kDa) was confirmed by 12% SDS-PAGE. The protein was crystallized from 6% Tacsimate pH 6.0, 0.1 M MES pH 6.0, 25% PEG 3350 with 100 mM NDSB 256 as an additive. The crystals diffracted to 2.5 Å resolution (Fig. 2) and belonged to the monoclinic space group $P2_1$, with unit-cell parameters $a = 77.9, b = 70.5, c = 126.5$ Å, $\beta = 106.2^\circ$. The Matthews coefficient was calculated to be

Figure 2
Diffraction image of an SSL1 crystal.

Figure 3
The self-rotation function plot of the SSL1 crystal shows two off-origin peaks suggesting the presence of two noncrystallographic twofold symmetry axes.

2.42 Å³ Da⁻¹, suggesting the presence of six molecules in the asymmetric unit (Matthews, 1968). The self-rotation function plot (Fig. 3) shows two off-origin peaks at $\chi = 180^\circ$, suggesting the presence of two noncrystallographic twofold symmetry axes. The structure was solved by molecular replacement using the crystal structure of staphylococcal superantigen-like protein 11 (PDB entry 2rdg; 43% identity; Chung *et al.*, 2007) as template with *MOLREP* (Vagin & Teplyakov, 2010). The final model building and restrained refinement using *REFMAC5* (Murshudov *et al.*, 2011) are currently in progress.

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