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Active site analysis of sortase A from *Staphylococcus simulans* indicates function in cleavage of putative cell wall proteins

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ABSTRACT

Sortase mediated transpeptidation reactions play a significant role in covalent attachment of surface proteins to the cell wall of Gram-positive bacteria. Earlier studies have shown that sortase A (StrA) is required for the virulence of *Staphylococci*. The human pathogen *Staphylococcus simulans* CJ16 carries a putative sortase A (SsiStrA) encoding gene, but neither transpeptidation activity nor biochemical characteristics of SsiStrA have been investigated. Here, we identified and characterized StrA from coagulase-negative *Staphylococci*. SsiStrA was cloned and overexpressed in *Escherichia coli* BL21 in a soluble form. Size-exclusion chromatography, cross-linking and dynamic light scattering demonstrated that SsiStrA existed as monomer-dimer equilibrium *in vitro*. We further demonstrated that SsiStrA has sortase activity, and it recognized and cleaved the sorting motif LXPTG. H117, C180 and R193 residues were critical for enzyme activity, and calcium ions enhanced activity.

1. Introduction

Many surface proteins of Gram-positive bacteria are covalently anchored to the cell wall by transpeptidase enzymes known as sortases, which were first identified in *Staphylococcus aureus* [1]. Since then, sortases have been identified in most Gram-positive bacteria, with many studies identifying and/or characterizing different types of sortases. So far, the sortases can be categorized based on their primary sequences and functions into six classes [2]. These enzymes are involved in several central processes of bacterial biology including adhesion, colonization, and biofilm formation [3].

Despite a large number of identified sortases, most biochemical investigations have been conducted with sortase A (SrtA). SrtA is usually found in a single copy per genome and plays a key role in the adhesion to and invasion of hosts [4]. It is responsible for anchoring most of the cell wall proteins to the cell wall. In the presence of Ca^{2+}, the active site cysteine of SrtA attacks the backbone carbonyl carbon of the threonine residue in the LPXTG motif, breaking the threonine and glycine peptide bond, which creates a thioester-linked acyl enzyme intermediate [3]. Many bacterial genomes encode more than one sortase and more than one LPXTG-containing protein [5]. Genome context in particular seems to be a strong indicator of functional relationship, as sortases and their substrates are often encoded in gene clusters on bacterial chromosomes.

Staphylococcus simulans is a member of coagulase-negative staphylococci (CoNS), which is part of the normal microbiota in animals and humans [6–8]. However, human infections with *S. simulans* are rarely reported. Previously, we described the first case of surgical site infection caused by a methicillin-resistant *S. simulans* (MRSS) isolate CJ16 [9]. However, in-depth analysis of potential colonization determinants and virulence factors for *S. simulans* has not been performed. The ability of *S. simulans* strains to adhere to epithelial cells is a crucial determinant of skin colonization and infection. Therefore, we hypothesize that *S. simulans* may have surface proteins for attachment that are anchored to the cell wall envelope by a mechanism similar to that observed for *S. aureus*. Research on sortases and their substrates is limited in

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CoNS, even though CoNS represent one of the major nosocomial pathogens [10]. Here, we first report the identification, purification and characterization of SrtA from CoNS. In addition, we demonstrate that Ca^{2+} stimulates the activity of SsiStrA catalyzed cleavage of LPETG.

2. Materials and methods

2.1. Strains and growth conditions

The clinical isolate *S. simulans* CJ16 was grown in Mueller-Hinton (MH) medium, *E. coli* DH5α and *E. coli* BL21 (DE3) (Novagen) were grown in Luria Bertani (LB) medium at 37 °C.

2.2. Homology searches

Search of the *S. simulans* CJ16 genome [9] for sortases and LPXTG-motif containing proteins was done using NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.html). Multiple alignment analysis was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/), and the result was analyzed with the program ESPript 2.2 (http://espript.ibcp.fr/ESPript/cgi-bin/ESPript.cgi) to generate alignment figure.

2.3. Homology modeling

The amino acid sequence of SsiStrA was submitted to SWISS-MODEL protein-modeling server with Automated Mode [11]. The 3-D models were subsequently analyzed and selected according to the best fit of the sequence identity. The best identity was the crystal structure of *S. aureus* StrA (PDBID: 1T2P), which was selected as the template of homology modeling for *S. simulans* StrA.

2.4. DNA cloning, site-directed mutagenesis and purification

The full length of *strA* was amplified from genomic DNA of CJ 16 with primers: (1) forward primer 5′-GGGAATTCcatatgATGGGTAAATGGAAATCACG-3′ and (2) reverse primer 5′-CCGctcgagCCGTTATAGCAACTCTTTCAGC-3′ (restriction sites are in small case). The PCR product was cloned into expression vector pET21a using restriction sites NdeI and XhoI to generate the pET21a-StrA plasmid. The expression plasmid pET21a-*strA* encoding wild-type *strA* served as a template for the introduction of single amino acid substitutions by PCR using the QuikChange method (Stratagene). Mutational changes were confirmed by DNA sequencing. The primers 5′-TCAATTGCAGGAgccACTTTCATTGAC-3′ and 5′-GTCAATGAAAGTagcTCCTGCAATTGA-3′(mutated bases are in small case) were used to generate the H117A mutation. Primers 5′-CATTAATTACTgctGATGATTACAATG-3′and 5′-CATTGTAATCATCagcAGTAATTAATG-3′were used to generate the C180A mutation. Primers 5′-GTTTGGGAAAAAgctAAAATCTTTGTAGC-3′ and 5′-GCTACAAAGATTTTagcTTTTTCCCAAC-3′were used to generate the R193A mutation. The identity of the DNA constructs was verified by DNA sequencing. The induced cells were harvested by centrifugation and re-suspended in a lysis buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM NaCl and 1 mM DTT. The contaminant proteins were removed with wash buffer containing 50 mM imidazole, and subsequently SsiStrA protein was eluted in elution buffer containing 100 mM imidazole.

2.5. Mass spectrometry

The overexpressed wild type SsiStrA protein was subjected to Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF/MS) analyses using ABI 4800 TOF-TOF Proteomics Analyzer (Applied Biosystems). To identify the protein, a peptide mass was searched against the reference peptides in the NCBI nr-database by using MASCOT software.

2.6. Analytical size-exclusion chromatography and circular dichroism spectroscopy

Gel filtration analysis for purified wild type SsiStrA protein was determined by size exclusion chromatography (SEC) as described previously [12]. The molecular mass standards were calibrated with ovalbumin (43 kDa), carbonic anhydrase (29 kDa), and ribonuclease (13.7 kDa), on an AKTA fast protein liquid chromatography system (GE healthcare). The peaks of interest were collected and confirmed with 12% SDS-PAGE.

Circular dichroism (CD) spectroscopy was carried out as described previously [13]. All the measurements have been repeated three times. CD experiments were conducted with a JASCO J-810 CD spectrometer equipped with a Jasco PFD-425S temperature control system. Wild type SsiStrA was dialyzed against buffer containing 25 mM Tris, 5 mM $CaCl_2$, and 75 mM aCl, pH 7.5. A 20 μL solution of 10 mM Na phosphate pH 7.5 was added to the dialyzed SrtA, which resulted in a 1 mL sample with a protein concentration of 9.8 μM. Data were collected at 25 °C and at 95 °C from 260 to 190 Nm.

2.7. In vitro chemical cross-linking and dynamic light scattering (DLS)

W carried out chemical cross-linking experiments with 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC; Sigma) as described before [14]. The purified SrtA protein was dissolved in 0.1 M 2-(4-morpholino) ethanesulfonic acid pH 4.5 to give a 2.5 mg/mL solution. This was added to a freshly prepared EDC aqueous solution (0.8 mg/mL). The final concentration of the proteins was 1.25 mg/mL. After 1 h at room temperature, the reaction was quenched with 2% (v/v) 2-mercaptoethanol. Samples were separated by 12% SDS-PAGE and visualized by Coomassie Blue staining.

DynaPro NanoStar (Wyatt Technology) was used to carry out DLS measurements. StrA in lysis buffer were centrifuged at 15,000 g for 30 min prior to measurements to remove dust and large aggregates. Measurements were performed at 25 °C in triplicate with at least 3 readings and an acquisition time of 8 s. The resulting distributions were derived from regularization fits to the average of 50 correlation curves using the DYNAMICS software (Wyatt) and are displayed as the intensity of light scattered as a function of the hydrodynamic radius.

2.8. Western blot analysis of proteins and immunofluorescence staining

Polyclonal anti-StrA antibodies were produced in rabbits using purified SiStrA (Genscript, China). For western blot analyses, cell lysates from the *S. simulans* CJ16 cell cultures and purified recombinant proteins were transferred to a nitrocellulose membrane. After blocking in PBST (PBS plus 0.05% Tween-20) with 5% skim milk powder for 2 h at room temperature, 1000-fold diluted rabbit anti-SrtA in PBST was added and incubated with continuous shaking for 1 h. AP-labeled goat anti-rabbit with 10,000-fold diluted in PBST was added. After 4 washes with PBST, NBT/BCIP substrate (BBI) was added to develop color.

2.9. Sortase activity measurements

Wild type SsiStrA and mutant proteins were assayed for activity

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