Engineering the specificity of *Streptococcus pyogenes* sortase A by loop grafting

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**Abstract**

Sortases are a group of enzymes displayed on the cell-wall of Gram-positive bacteria. They are responsible for the attachment of virulence factors onto the peptidoglycan in a transpeptidation reaction through recognition of a pentapeptide substrate. Most housekeeping sortases recognize one specific pentapeptide motif; however, *Streptococcus pyogenes* sortase A (SpSrtA WT) recognizes LPETG, LPETA and LPKLG motifs. Here, we examined SpSrtA's flexible substrate specificity by investigating the role of the β7/8 loop in determining substrate specificity. We exchanged the β7/8 loop in SpSrtA with corresponding β7/8 loops from *Staphylococcus aureus* (SaSrtA WT) and *Bacillus anthracis* (BaSrtA WT). While the BaSrtA-derived variant showed no enzymatic activity toward either LPETG or LPETA substrates, the activity of the SaSrtA-derived mutant toward the LPETA substrate was completely abolished. Instead, the mutant had an improved activity toward LPETG, the preferred substrate of SaSrtA WT.

**KEYWORDS**

computational analysis, loop swapping, protein engineering, *Streptococcus pyogenes* sortase A, substrate specificity

1 | INTRODUCTION

Gram-positive bacteria display proteins on their surface, which help them interact with the environment. These surface proteins, often virulence factors, are attached to the outer envelope of Gram-positive bacteria via a transpeptidation reaction catalyzed by sortases; these enzymes recognize and break the peptidoglycan to create a new peptide bond. Based on sequence alignments and predicted substrate preferences, the sortase superfamily has been divided into six classes A-F. Different 3D structures have revealed a common eight-stranded β-barrel "sortase fold," providing details on the active site environment and the catalytic triad of Cys, His, and Arg. In the proposed model for the catalytic mechanism, the catalytic Cys residue is in a deprotonated state, whereas the His residue occurs in a protonated form. Upon binding of the substrate, the thiolate of the Cys attacks the carbonyl group of Thr in the substrate and thus forms a tetrahedral intermediate. The His residue on the other hand is hypothesized to be involved in the protonation of the substrate leaving group, which leads to the formation of an acyl-enzyme intermediate. The function of the Arg residue in the transpeptidation reaction performed by sortases is still poorly understood, though it is thought it might aid in the stabilization of the acylated product.
However, some significant variations within the catalytic centers of different sortases have been observed. For the best studied sortases, which belong to class A, the main differences around the conserved catalytic domain have been described for the area of the N-terminus that precedes the catalytic domain, the β6/β7 loop, the β7/β8 loop and the C-terminus of the protein. So far, the most information regarding the structure and catalytic mechanism of the sortase superfamily has been obtained from studies on the Staphylococcus aureus sortase A (SaSrtA WT). Here, we focus on the less explored, homologous sortase A from Streptococcus pyogenes (SpSrtA WT), which exhibits certain differences in substrate profile and structure. Unlike SaSrtA WT, the SpSrtA WT can recognize not only the canonical LPXTG (X being any amino acid) pentapeptide motif but also LPXTA and LPKLG motifs. This allows a somewhat broader scope of substrates for sortase-mediated ligation.

The structure of SpSrtA WT exhibits some notable differences around the active site, which distinguish it from SaSrtA WT: (a) no Ca²⁺ binding site for allosteric activation, (b) a channel that leads to the active site of the enzyme, and (c) an opened β7/β8 loop, creating a prolonged groove. Since the β7/β8 loop of SaSrtA WT is involved in the interaction with the C-terminal part of the LPXTG substrate and the incoming nucleophile, we hypothesized that the opened β7/β8 loop of the SpSrtA WT plays an important role in this enzyme’s broader substrate specificity. Therefore, we designed a loop hybrid based on the scaffold sequence of SpSrtA WT (PDB 3FN5) grafted with the β7/β8 loop from SaSrtA WT (PDB 2RUI). Indeed, we found that replacement of the SpSrtA loop led to a shift in substrate preference of this variant toward LPXTG while abolishing activity toward LPXTA. We also created a second loop variant using the β7/β8 loop from Bacillus anthracis sortase A (BaSrtA WT, PDB 2RUI). This loop is comparable in size to that of SpSrtA WT but differs in dynamics: it undergoes a disordered to ordered transition after binding of the substrate. Replacement of the β7/β8 loop led to inactivation of the BaSrtA-derived variant. Overall, our work provides insight into the flexible substrate specificity of SpSrtA.

2 MATERIALS AND METHODS

2.1 Computational analysis of the SrtA structures

For the identification of the residues in the β7/β8 loops intended for grafting, we constructed a structure-based alignment using the constraint-based multiple alignment tool (COBALT), available on the National Center for Biotechnology Information (NCBI) website. The results were downloaded in FASTA format and analyzed further using the Jalview software. A superimposition of the SrtA enzymes (PDB 3FN5 with 2KID, and with 2RUI) was generated using the 3DMA module within the BIOVIA Discovery Studio software. Based on these superimposition studies, we decided to swap three fragments within the region of the β7/β8 loops: the fragment I211-E215 from SpSrtA WT was selected to be exchanged with Y187-K196 from SaSrtA WT (Sp_LoSa) and with V190-K195 from BaSrtA WT (Sp_LoBa). Additionally, we exchanged the β7/β8 loop of SaSrtA WT (Y187-K196) for the fragment I211-E215 from SpSrtA WT, thus creating mutant Sa_LoSp (Figure S1).

The LPETG substrate was modeled into the structure of the SpSrtA WT and the model of the SaSrtA-derived variant (Sp_LoSa). The model of the enzyme-substrate complex was generated using data obtained from the 3D structure of SaSrtA WT covalently bound with an LPXTG analog (2KID) and known features of the SpSrtA WT enzyme. To further optimize the docking of the substrate we minimized the energy using the Smart Minimizer protocol from the BIOVIA Discovery Studio software. The protocol was set to a maximum of 200 steps and an RMS gradient tolerance of 0.1 kcal/(mol × Å).

2.2 Generation of the loop mutants

The gene encoding truncated SpSrtAΔΔBT WT (kindly provided by Dr M. J. Banfield, Newcastle University, UK) was cloned into plasmid pQIq17 between the BamHI and HindIII sites and subsequently used as a template for the preparation of the loop mutants. Using AQUA cloning, DNA encoding position I211-E215 of the SpSrtA β7/β8 loop was exchanged with DNA encoding positions Y187-K196 (SaSrtA β7/β8 loop) and V190-K195 (BaSrtA β7/β8 loop), and the SaSrtA β7/β8 loop was exchanged with DNA encoding positions I211-E215 (SpSrtA β7/β8 loop) (primer sequences in Table S1). Escherichia coli turbo competent cells (New England Biolabs) were used for cloning and grafting was confirmed by DNA sequencing.

2.3 Protein production and Purification

Production and purification of proteins used in this study were performed as described previously. Briefly, competent E. coli BL21(DE3) cells were transformed with plasmids encoding SpSrtA WT and loop mutants. Overnight cultures were used to inoculate 1 L of 2 x YT media supplemented with 100 μg/mL ampicillin. Protein production was induced with the addition of IPTG to a final concentration of 1 mM (Duchefa, The Netherlands) and continued for 4 hours at 37°C with orbital shaking (200 rpm). Next, cultures were centrifuged and cell pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl and 10 mM imidazole. Cells were disrupted by sonication and the clarified lysates were used for affinity purification via the N-terminal His-tag. Proteins were purified to 90% purity by preparative size-exclusion chromatography on a Superdex75 16/60 column (GE Healthcare).

2.4 Thermal denaturation measurement

The unfolding of the SpSrtA WT and mutants was analyzed with differential scanning fluorimetry (DSP) using a CFX96 Touch Real-Time PCR Detection system (Bio-Rad). Proteins at a concentration of 1 mg/mL were mixed with the SYPRO Orange dye (Sigma-Aldrich)
according to the manufacturer’s protocol. The fluorescence signal was continuously measured at the emission wavelength of 556 nm, with the temperature increasing from 20°C to 70°C (1°C/minute). Assuming a two-state model for protein denaturation, the fraction of folded protein ($P_f$), the melting temperature of the proteins and nonlinear fitting of the Boltzmann’s sigmoidal equation were calculated as reported before using GraphPad Prism.

### 2.5 Activity measurement

The activity of WT enzymes and their mutants was measured using a fluorometric assay with quenched substrate analogs Abz-LPET-A-Dap (Dnp) and Abz-LPETG-A-Dap(Dnp) (Bachem AG, Switzerland). After cleavage of the quencher, the increase in fluorescence (excitation wavelength 355 nm) was recorded at emission wavelength 460 nm. Measurements were performed using a FLUOstar Omega spectrometer (BMG LABTECH). Enzyme concentrations were kept at 2 μM in a final reaction volume of 100 μL. The reaction buffer was composed of 50 mM Tris-HCl, pH 7.5, supplemented with 150 mM NaCl.

### Substrates and nucleophiles were added to the reaction to a final concentration of 20 μM and 2 mM, respectively. The data in this study are reported as the slope values obtained from the linear phase of the cleavage reaction.

### 3 RESULTS

#### 3.1 Identification of residues in the β7/β8 loops intended for grafting

SpSrtA WT, SaSrtA WT, and BaSrtA WT share the conserved “sortase fold,” with a few alterations observed in this study (Figure 1). The sequence identity between SpSrtA and SaSrtA is 29% and between SpSrtA and BaSrtA is 32%. The β7/β8 loop of the SpSrtA WT is comparable in size to the loop in BaSrtA WT, yet much smaller and more rigid compared to the β7/β8 loop of the SaSrtA WT. Since the SaSrtA WT enzyme is to date the best characterized sortase, it was used as a template for the localization of the β7/β8 loops in the analyzed structures. The average distance between...
the atoms of the superimposed enzymes used in this study was calculated as the root-mean-square deviation (RMSD). SpSrtA WT and SaSrtA WT superimposed with an RMSD of 1.3 Å, whereas SpSrtA WT and BaSrtA WT superimposed with an RMSD of 1.5 Å. The cut-off for the distance of consecutive Cα atoms was set at 2.5 Å. Based on the structural alignments and superimposition studies (Figure 1), we chose stretches of residues located in the β7/β8 loops (Table 1) for exchange. The β7/β8 loop in the structure of SaSrtA WT selected for grafting is five amino acids longer than the β7/β8 loop of SpSrtA WT. The β7/β8 loops in SpSrtA WT and BaSrtA WT represent relatively short fragments composed of five and six amino acids (Table 1), respectively.

Since the catalytic Cys residue for each of these enzymes is located within the β7/β8 loop, grafting was performed with a two amino acids’ distance from the catalytic center.

### 3.2 Differential scanning fluorimetry analysis of SpSrtA WT and mutants

Every modification introduced into the structure of a protein may cause changes in the secondary structure and the folding of the protein. Particularly larger changes such as loop grafts may lead to protein misfolding. Therefore, DSF was used to assess the thermal transition from the folded to the unfolded state of the SpSrtA WT and the mutants. The results of protein unfolding upon temperature increase are shown in Figure 2.

As shown in Figure 2, all enzymes examined exhibited a sigmoidal transition from the native state to unfolded protein when exposed to increasing temperature. The melting temperatures (Tm) were calculated as described in section 2 and are given in Table 2. Both mutants of SpSrtA WT showed slightly increased Tm values in comparison to the WT.

### 3.3 Activity measurement of SpSrtA WT and mutants

In order to estimate the effect of replacement of the β7/β8 loop on the substrate specificity and activity of the enzyme, we performed activity measurements as described in section 2. Both LPETA and LPETG substrate analogs were tested in combination with the nucleophiles 2-Ala and 5-Gly, respectively. The result of these activity measurements is shown in Figure 3.

Although the created mutants were properly folded (Figure 2), activity was only measured for the variant with the loop fragment derived from SaSrtA WT. Conversely, a Sa_LoSp mutant (SaSrtA with the loop from the SpSrtA WT) did not show proper unfolding using DSF (data not shown) nor did it show enzymatic activity in the fluorescence assay (Figure S1), indicating the enzyme is nonfunctional. In our study, the SpSrtA WT showed higher activity toward the LPETA substrate analog than toward LPETG. In the case of the Sp_LoBa mutant, no activity toward either LPETA or LPETG could be measured (Figure 3). Interestingly, while the activity of the Sp_LoSa mutant was completely abolished for the LPETA substrate in combination with the 2-Ala nucleophile, activity toward the LPETG substrate was maintained and even slightly improved, suggesting that the mutant did indeed acquire an LPETG substrate preference like SaSrtA WT. To learn more about the difference in the location of the loop β7/β8 of the SpSrtA WT and the Sp LoSa in reference to the LPETG substrate, we looked at the superimposition of SpSrtA WT (PDB 3FN5) and a model of SpLoSa (Figure 4). One of the main differences we noticed was the presence of a Trp residue in the β7/β8 loop of the Sp_LoSa mutant, which is positioned very closely to the substrate groove (Figure 4, shown in blue).

### Table 1 Amino acid sequences and their locations in the β7/β8 loops used for the mutagenesis studies performed in this study

| Enzyme   | Origin of grafted loop | Residues (original position) |
|----------|------------------------|-----------------------------|
| SpSrtA WT | —                      | IEATE (211-215)             |
| Sp_LoSa  | SaSrtA WT              | YNEKTGWEK (187-196)        |
| Sp_LoBa  | BaSrtA WT              | VKDNSK (190-195)           |

### Table 2 The Tm values for SpSrtA WT and the two loop mutants, Sp_LoSa and Sp_LoBa

| Enzyme   | Tm (°C) |
|----------|---------|
| SpSrtA WT | 65 ± 0.2 |
| Sp_LoSa  | 68 ± 0.5 |
| Sp_LoBa  | 66 ± 0.7 |
aforementioned Trp residue in the Sp_LoSa mutant hinders the accessibility to the active site for substrates terminating in residues other than Gly.

4 | DISCUSSION

Evolution of proteins in nature does not only happen by means of single-point modifications, sometimes bigger fragments such as loops and domains are exchanged. Currently, this exchange of fragments can be rationally applied in the engineering of a protein’s biochemistry or modification of their features. The approach makes use of existing protein scaffolds in which (large) fragments of proteins are exchanged to design proteins with potentially new, unnatural folds and with improved functions. Domain and loop swapping has been applied successfully in the engineering of many different features of enzymes and proteins, such as the change of catalytic activity of glyoxalase II, the inversion of enantioselectivity of Bacillus subtilis Lipase A, and the humanization of antibodies.

Loop swapping was also applied to SaSrtA WT in order to better understand substrate recognition. Previous work on SaSrtA WT showed that this active site loop is involved in the interaction with the substrate, the β6/β7 loop undergoes a disorder-to-order transition after binding of a single Ca²⁺ ion, which then promotes the binding of the substrate. SaSrtA’s β6/β7 loop was exchanged for the corresponding loop from the S aureus sortase B (SaSrtB). Once the β6/β7 loop from the SaSrtB was grafted onto the SaSrtA WT, the substrate specificity of the SaSrtA WT was switched to an NPQTN substrate, which is characteristic for class B sortases. This study confirmed that the β6/β7 loop of the SaSrtA WT makes an important site for substrate recognition and also showed that the extended β6/β7 loop of the SaSrtB determines the recognition of the NPQTN motif characteristic for class B sortases.

In fact, one of the most studied regions of sortase A enzymes with known 3D structures is the β7/β8 loop. For BaSrtA WT, the β6/β7 loop undergoes a similar transition as SaSrtA WT before binding of the substrate, though it is a Ca²⁺-independent enzyme. Although sortases share the same eight-stranded β-barrel fold, recognition of the substrate may be modulated by different parts of the enzyme. Some studies revealed that N-terminal helices may modulate substrate binding. Weiner et al. found that the N-terminal appendage of BaSrtA, which consists of 23 amino acids, is responsible for partial shielding of the active site and, as a consequence, regulation of substrate access. This feature may aid in the reduction of unwanted hydrolytic cleavage. A similar structural feature was observed for Streptococcus mutans SrtA, where the N-terminal appendage was found to interact with the active site of the enzyme.

Our study focused on the β7/β8 loop, which we hypothesized to be involved in the more flexible substrate specificity of SpSrtA WT. Previously elucidated 3D structures of sortases from class A revealed that a displaced β7/β8 loop plays a role in the formation of a second groove located near the active site. For example, this behavior was observed for BaSrtA, for which the binding of the substrate leads to transition of the β7/β8 loop, which then forms a surface for the transpeptidation reaction. For our grafting experiments, we chose two enzymes with known 3D structures, SaSrtA WT and BaSrtA WT. Although their β7/β8 loops present different lengths...
and dynamics, these enzymes are known to be highly specific toward the LPXTG substrate. The presence of a slightly bigger amino acid in the motif, LPXTA, abolishes the enzymatic activity in an in vitro environment.\textsuperscript{14} SpSrtA WT on the other hand can accept more substrates: the motif, LPXTA, abolishes the enzymatic activity in an in vitro environment.\textsuperscript{14} SpSrtA WT resulted in a change in substrate preference. Along with the introduction of the β7/β8 loop from SaSrtA WT into the structure of SpSrtA WT, the specificity of the Sp LoSa mutant became exclusively directed toward LPETG substrate, similar to SaSrtA WT (Figure 3). In the superimposition model of SpSrtA WT and the Sp LoSa mutant (Figure 4), we noticed the presence of an aromatic residue located near the active site of the mutant. We speculate that this Trp residue plays a key role in regulating the enzyme’s specificity by physically blocking access to the substrate groove.\textsuperscript{12} In contrast, after the introduction of the loop from BaSrtA WT with a similar length but different amino acid composition, the resulting Sp LoBa mutant had lost its activity (Figure 3); nevertheless, the enzyme was properly folded (Figure 2).

Engineering of the enzyme specificity can be difficult due to a variable number of modifications that need to be introduced into the structure of enzymes in order to change substrate preference. For some enzymes, it is sufficient to introduce a single mutation in order to change its substrate specificity.\textsuperscript{22} However, other enzymes require more advanced modifications such as the exchange of whole domains between homologous enzymes. Here, we highlighted the less studied β7/β8 loop from SrtA enzymes and its significance in substrate recognition. Our results indicate that the β7/β8 loop regulates substrate access to the active site and would therefore, along with the β6/β7 loop, form a compelling starting point to engineer the specificity of sortase enzymes.

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REFERENCES

1. Mazmanian SK, Liu G, Ton-That H, Schneewind O. Staphylococcus aureus Sortase, an enzyme that anchors surface proteins to the cell wall. Science. 1999;285(5428):760-763.
2. Cascioferro S, Totsika M, Schillaci D. Sortase A: an ideal target for anti-virulence drug development. Microb Pathog. 2014;77:105-112.
3. Bradshaw WJ, Davies AH, Chambers CJ, Roberts AK, Shone CC, Acharya KR. Molecular features of the sortase enzyme family. FEBS J. 2015;282(11):2097-2114.
4. Dramsi S, Trieu-Cuot P, Bierne H. Sorting sortases: a nomenclature proposal for the various sortases of gram-positive bacteria. Res Microbiol. 2005;156(3):289-297.
5. Spirig T, Weiner EM, Clubb RT. Sortase enzymes in gram-positive bacteria. Mol Microbiol. 2011;82(5):1044-1059.
6. Frankel BA, Kruger RG, Robinson DE, Kelleher NL, McCafferty DG. Staphylococcus aureus sortase transpeptidase SrtA: insight into the kinetic mechanism and evidence for a reverse protonation catalytic mechanism. Biochemistry. 2005;44(33):11188-11200.
7. Zong Y, Bice TW, Ton-That H, Schneewind O, Narayana SVL. Crystal structures of Staphylococcus aureus Sortase a and its substrate complex. J Biol Chem. 2004;279(30):31383-31389.
8. Jacobitz AW, Kattke MD, Weresszczynski J, Clubb RT. Sortase Transpeptidases: structural biology and catalytic mechanism. Adv Protein Chem Struct Biol. 2017;109:223-264.
9. Race PR, Bentley ML, Melvin JA, et al. Crystal structure of Streptococcus pyogenes Sortase A: implications for sortase mechanism. J Biol Chem. 2009;284(11):6924-6933.
10. Schmohl L, Bierneier J, von Kügelgen N, et al. Identification of sortase substrates by specificity profiling. Bioorganic Med Chem. 2017;25(18):5002-5007.
11. Nikghalb KD, Horvath NM, Prelesnik JL, et al. Expanding the scope of Sortase-mediated ligation by using Sortase homologues. Chembiochem. 2018;19(2):185-195.
12. Suree N, Liew CK, Villareal VA, et al. The structure of the Staphylococcus aureus sortase-substrate complex reveals how the universally conserved LPXTG sorting signal is recognized. J Biol Chem. 2009;284(36):24465-24477.
13. Chan AH, Yi T, Terwilliger AL, Maresso AW, Jung ME, Clubb RT. Structure of the Bacillus anthracis Sortase a enzyme bound to its sorting signal: a flexible amino-terminal appendage modulates substrate access. J Biol Chem. 2015;290(42):25461-25474.
14. Weiner EM, Robson S, Marohn M, Clubb RT. The sortase a enzyme that attaches proteins to the cell wall of Bacillus anthracis contains an unusual active site architecture. J Biol Chem. 2010;285(30):23433-23443.
15. Papadopoulos JS, Agarwala R. COBALT: constraint-based alignment tool for multiple protein sequences. Bioinformatics. 2007;23(9):1073-1079.
16. Waterhouse AM, Procter JB, Martin DMA, Clamp M, Barton GJ. Jalview version 2-a multiple sequence alignment editor and analysis workbench. Bioinformatics. 2009;25(9):1189-1191.
17. Simon M, Zangemeister-Wittke U, Plückthun A. Facile double-functionalization of designed Ankyrin repeat proteins using click and thiol chemistries. Bioconjug Chem. 2012;23(2):279-286.
18. Beyer HM, Gonschorak P, Samodelov SL, Meier M, Weber W, Zurbriggen MD. AQUA cloning: a versatile and simple enzyme-free cloning approach. PLos One. 2015;10(9):1-20.
19. Wójcik M, Elefteriadis N, Zwinderman MRH, Dömling ASS, Dekker FJ, Boersma YL. Identification of potential antivirulence agents by substitution-oriented screening for inhibitors of Streptococcus pyogenes sortase a. Eur J Med Chem. 2019;161:93-100.
20. Rosa N, Ristic M, Seabrook SA, Lovell D, Lucent D, Meltdown NJ. A tool to help in the interpretation of thermal melt curves acquired by differential scanning fluorimetry. J Biomol Screen. 2015;20(7):898-905.
21. Huysh K, Partch CL. Analysis of protein stability and ligand interactions by thermal shift assay. Curr Protoc Protein Sci. 2015;79:28.9.1-28.9.14.
22. Ton-That H, Mazmanian SK, Faull KF, Schneewind O. Anchoring of surface proteins to the cell wall of Staphylococcus aureus. J Biol Chem. 2000;275(13):9876-9881.
23. Pang X, Zhou HX. Disorder-to-order transition of an active-site loop mediates the allosteric activation of Sortase a. Biophys J. 2015;109(8):1706-1715.
24. Marraffini LA, DeDent AC, Schneewind O. Sortases and the art of anchoring proteins to the envelopes of gram-positive Bacteria. Microbiol Mol Biol Rev. 2006;70(1):192-221.
25. Shapiro J. Genome organization, natural genetic engineering and adaptive mutation. Trends Genet. 1997 Mar;13(3):98-104.
26. Bogarad LD, Deem MW. A hierarchical approach to protein molecular evolution. *Proc Natl Acad Sci*. 2002;99(6):2591-2595.
27. Park HS, Nam SH, Lee JK, et al. Design and evolution of new catalytic activity with an existing protein scaffold. *Science*. 2006;311(5760):535-538.
28. Tawfik DS. Loop grafting and the origins of enzyme species. *Sci Biochem*. 2006;311(5760):475-476.
29. Boersma YL, Pijning T, Bosma MS, et al. Loop grafting of *Bacillus subtilis* lipase a: inversion of Enantioselectivity. *Chem Biol*. 2008;15(8):782-789.
30. Kashmiri SVS, De Pascalis R, Gonzales NR, Schlom J. SDR grafting - a new approach to antibody humanization. *Methods*. 2005;36(1):25-34.
31. Bentley ML, Gaweska H, Kielec JM, McCafferty DG. Engineering the substrate specificity of *Staphylococcus aureus* sortase a: the β6/β7 loop from SrtB confers npqtn recognition to SrtA. *J Biol Chem*. 2007;282(9):6571-6581.
32. Naik MT, Suree N, Ilangovan U, et al. *Staphylococcus aureus* sortase a transpeptidase: calcium promotes sorting signal binding by altering the mobility and structure of an active site loop. *J Biol Chem*. 2006;281(3):1817-1826.
33. Wallock-Richards DJ, Marles-Wright J, Clarke DJ, et al. Molecular basis of *Streptococcus mutans* sortase a inhibition by the flavonoid natural product trans-chalcone. *Chem Commun*. 2015;51(52):10483-10485.
34. Khare B, Krishnan V, Rajashankar KR, et al. Structural differences between the *Streptococcus agalactiae* housekeeping and pilus-specific sortases: SrtA and SrtC1. *PLoS One*. 2011;6(8):e22995.
35. Zong Y, Mazmanian SK, Schneewind O, Narayana SVL. The structure of Sortase B, a cysteine Transpeptidase that tethers surface protein to the *Staphylococcus aureus*. *Cell Wall Struct*. 2004;12(1):105-112.
36. Bozkurt G, Ploegh HL, Kundrat L, et al. Site-specific C-terminal and internal loop labeling of proteins using sortase-mediated reactions. *Nat Protoc*. 2013;8(9):1787-1799.
37. Shi D, Yu X, Cabrera-Luque J, et al. A single mutation in the active site swaps the substrate specificity of N-acetyl-L-ornithine transcarbamylase and N-succinyl-L-ornithine transcarbamylase. *Protein Sci*. 2007;16:1689-1699.

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