# Pathogenic, versatile and tunable activity of sortase, a transpeptidation machine

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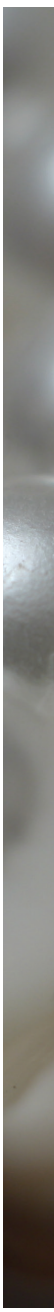
# SUMMARY, GENERAL DISCUSSION AND FUTURE OUTLOOK



Bacterial sortases are a very interesting but also challenging subject of study. The whole family consists of six dissimilar groups and each group consists of hundreds of different members. Despite general knowledge about sortases, coming mostly from studies performed on *Staphylococcus aureus* sortase A (SrtA), every member of the sortase family needs to be examined separately with respect to specificity and activity. Additionally, sortases are a group of enzymes with two sides to them, as they can be used as a target for new antibiotic strategies and as a tool for biotechnological applications. Such abilities leave the door wide open to a great range of studies that can be performed on this enzyme; work performed in this thesis focused on SrtA from two strains of Gram-positive bacteria, *Staphylococcus aureus* and *Streptococcus pyogenes.* I examined the potential of SrtA as a target for antimicrobial drugs and as a tool for site-specific conjugations. While exploring and optimizing features of SrtA, I made use of different protein engineering techniques. Additionally, I looked at the recent advances in high-throughput screening systems and applied one of these methods to the evolution of SrtA from *S. aureus*.

**Sortase as a target**

Gram-positive microorganisms, in particular bacteria belonging to a group of Gram-positive cocci, pose a serious threat to human health. The wide spectrum of diseases they are responsible for includes pneumonia, toxic shock syndrome, skin infections and meningitis. One of the main differences between Gram-positive and Gram-negative bacteria is the composition of the polymeric layer outside the plasma membrane, the peptidoglycan. Gram-positive bacteria possess a much thicker peptidoglycan layer than the Gram-negative bacteria. Among the molecules attached to the peptidoglycan are several virulence factors – proteins that play a role in the host invasion, colonization and development of infection. Although virulence factors vary greatly in their function and purpose, most of them need to undergo the same transpeptidation reaction before they are successfully attached to the peptidoglycan layer. The “machinery” responsible for this reaction consists of members of the sortase superfamily that can be found in most strains of Gram-positive bacteria.

Until now, gene knockout studies conducted on mouse models proved that *S. aureus* mutant strains lacking the *srtA* gene are deprived of the initiation of infections. That is due to the lack of surface protein display of virulence factors. Also, studies performed on *S. pyogenes* showed that a *srtA* knockout strain is susceptible to phagocytic killing. Mutants lacking the SrtA enzyme did not show growth defects suggesting that this enzyme is not essential for the life processes of bacteria. Hence,

using sortases as a target for new antibiotic therapies could place less selective pressure on bacterial survival, thus lowering the potential for antibiotic resistance.

**Chapter 2** focuses on the exploration of SrtA from *S. pyogenes* as a therapeutic target for antimicrobial drug development. For this, a library of nitrogen-containing aromatic compounds with different substitution patterns was designed and screened for a potent inhibitor. The most promising inhibitor, C10 with an $IC_{50}$ = 10 μM, was selected for further studies, which comprised the determination of the mechanism of inhibition and structure-activity relationships. Interestingly, the inhibitor C10 showed high specificity towards *S. pyogenes* SrtA while not inhibiting the related SrtA from *S. aureus*. Although the effect of inhibition of SrtA was presented only in an *in vitro* environment we believe that this indole-based compound can aid in further exploration of sortase inhibitors.

**Sortase as a tool**

Enzyme engineering is the alteration of the genetic information of enzymes which leads to modification or enhancement of their features and applications. There are several methods which are used in this field, including site-directed mutagenesis and chemical modification. However, to find the best variant(s) from a pool of enzyme variants, it is important to have a reliable method for the selection of residues destined for modification as well as a suitable screening platform. For over thirty years enzyme engineering brought not only a lot of proteins with improved characteristics but also advances in the process of engineering them.



The biggest part of this thesis revolves around engineering aspects of SrtA. The sortase-mediated transpeptidation reaction, also called sortagging, has great potential to be used in a variety of biotechnology-based applications: e.g., labelling or modification of recombinantly expressed proteins and proteins on the surface of living cells, and covalent anchoring of proteins to solid supports. After production of soluble, truncated SrtA from *S. aureus* and *S. pyogenes* the transpeptidation reaction has been successfully used *in vitro*. Nevertheless, the catalytic efficiency of sortases is very poor and therefore lowers the potential of sortagging.

As demonstrated in **Chapters 3, 4** and **5**, protein engineering approaches, both rational and random, were successfully applied to improve SrtA characteristics. The aim of **Chapter 3** was the improvement of *S. pyogenes* sortase A activity and the broadening of its substrate specificity. Using a semi-rational approach consisting of Rosetta modeling and iterative saturation mutagenesis (ISM), a triple SrtA mutant

with a twofold increased affinity in the recognition of the LPETA substrate compared to the wild-type enzyme was found. Additionally, in **Chapter 4** loop grafting studies were performed on *S. pyogenes* SrtA. The study provided insight into the design and function of the β7/β8 loop of this enzyme. Indirectly, this study confirmed the importance of a few amino acids located in the β7/β8 loop of the *S. aureus* SrtA, from which the loop in the grafting experiments was derived. Unfortunately, the crystal structure of the SpSrtA WT has only been solved for the free enzyme without the substrate analogue (PDB 3FN5). Information about the behavior of the SpSrtA WT in the presence of the substrate came from modeling studies as well as from engineering studies utilizing loop swapping.

The evolution of protein thermostability is a complex process composed of different evolutionary pathways that lead to an increased stability. In other words, homologous proteins found in two organisms that reside in different environments can adjust to the conditions encountered using different mechanisms of stabilization, thus generating variable modifications in its structure. The focus of **Chapter 5** was on the exploration and modification of the *S. aureus* SrtA in terms of thermostability. The enzyme was subjected to a consensus design approach; from this approach, specific mutations were introduced into the enzyme and their effect on thermostability of SrtA was evaluated using circular dichroism spectroscopy (CD). Two mutations, M155V and V193R, were found to increase the thermostability of SrtA and improve the enzyme's activity. When combined with a known pentamutant, M155V also conferred $Ca^{2+}$-independence to the enzyme. This study showed how the consensus design could be applied for the improvement of SrtA stability. We envision, variants obtained in this study could be used to perform the conjugation reaction in a low $Ca^{2+}$, intracellular environment.

Since library screening for mutants with desired properties requires a significant amount of time and resources, **Chapter 6** provides an overview of two high-throughput techniques that emerged in the field of protein engineering together with the development of compartmentalization: fluorescence-activated cell sorting (FACS) and microfluidics. The review focuses on advances in the application of these methods in the context of protein engineering, with an emphasis on applications in enzyme engineering. An example of a FACS-based technique, the so-called cellular encapsulation solubilization and screening (CHESS), and its application in sortase engineering is described in **Chapter 7**. This chapter explores the implementation of the CHESS technique to shorten the time of screening of the libraries of SrtA mutants. Although the encapsulation process was successful, the standardization

of this method for the evolution of SrtA turned out to be more challenging than expected: the transpeptidation product could not be detected by FACS, nor by confocal microscopy.

**Future outlook**

Now that the molecular functions of sortases have been elucidated, new studies towards the use of this knowledge in developing novel antimicrobials and novel diagnostic tools have got off the ground. The sortase superfamily represents a unique novel target for antibiotics and the search for potent inhibitors of this enzyme is at its start. This thesis showed that smart screening of SrtA from *S. pyogenes* in inhibition studies has resulted in the discovery of a potent inhibitor. Nevertheless, the inhibition of SrtA was only shown in an *in vitro* environment. For establishment of the inhibitor's antimicrobial action *in vivo* studies on *S. pyogenes* cells are needed. Furthermore, introduction of an infection model such as *Galleria mellonella* to the study of SrtA inhibition could help with better understanding the interaction between small molecule inhibitors and the SrtA enzyme in a much more natural environment. Although the selected small molecule showed the best solubility properties in comparison to other compound hits, the ability of the compound to get across the peptidoglycan layer and to reach sortase remains to be investigated. Finally, co-crystallization of the enzyme with the inhibitor(s) would give a better picture of the interaction between the enzyme and the molecule(s) and would form a lead in exploring existing and new sortase inhibitors. Finally, the species specificity of inhibitors should give an insight in the potential spectrum of pathogens that can be targeted with sortase inhibitors.



With respect to the engineering of sortases for transpeptidation reactions, already some work has been done, but there is still room for improvement. Future directions concern broadening of substrate specificity and the number of acceptor sequences recognized by sortase. This could lead to the development of novel protein fusions, e.g., branched polypeptides, polyproteins, cyclic peptides and new or improved applications in the *in vivo* protein ligation or chemoenzymatic site-specific labeling of histological samples. Also, a better control over the reaction conditions could minimize the effect of side reactions where other nucleophiles than the desired one compete during the transpeptidation reaction performed by sortase. Going forward, this could lower the amount of reaction components needed for the optimal performance of transpeptidation reaction. Last but not least, apart from SrtA, there are a number of other members of the sortase family, which could have potential for site-specific conjugation reactions. For instance, sortases belonging

to class C, which are responsible for the assembly of pili, have the ability to form isopeptide bonds. One of the applications of this reaction could be the broadening of site-specific antibody-drug conjugation and dual site-specific labeling of proteins.

Naturally, a simple and high-throughput technique for screening of all generated mutants could improve the process of engineering SrtA even further. For this, work on the development of new methods based on flow cytometry or microfluidics is needed. One of the pivotal parts of this progress is the advancement of compartmentalization techniques. Inspired by nature's own compartments such as prokaryotic or eukaryotic cells several strategies evolved. One of the approaches was generation of man-made polymer-based compartments called cellular high-throughput encapsulation, solubilization and screening (CHESS). So far, this method was successfully employed for the evolution of transmembrane receptors and soluble proteins but not enzymes. Since SrtA is a relatively small enzyme, in future experiments it might require additional preparation in order to be big enough to remain inside the capsules. Moreover, the choice and delivery of the suitable components of reaction to the capsule is another challenging aspect for the set-up of CHESS methodology.

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