Chapter 1

General introduction, aim and outline of the thesis
**GENERAL INTRODUCTION**

*Staphylococcus aureus* (*S. aureus*) is a Gram-positive bacterium, which is named after its appearance under the microscope (‘*staphylococcus*’, a bunch of grapes) and on solid media (‘*aureus*’, golden) [1]. *S. aureus* is a common human pathogen and can unnoticeably persistently colonize approximately 20% of the human population [2]. Colonized individuals (carriers) have an increased risk to develop *S. aureus* infections over non-colonized individuals (non-carriers) [2]. *S. aureus* infections rates are also higher among patients with type 1 diabetes [3], surgical patients [4,5], patients with immunodeficiency [6], patients undergoing hemodialysis [7] and drug users [8].

The infection spectrum of *S. aureus* with its incidence is diverse: skin and soft tissue infections (with approximately 700,000 admissions yearly in the U.S.) [9], bacteremia (incidence of 10-30 per 100,000 person-years) [10], infective endocarditis (*S. aureus* causes 16-34 % of all cases) [11,12], osteomyelitis (*S. aureus* causes 55 % of all cases) [13], pleuropulmonary infections (*S. aureus* accounts for > 40 % of health care-associated pneumonia cases in the U.S.) [14], and medical device-related infections, such as infected joint replacements (up to 18-73 % of all cases) [14]. The cure rate of staphylococcal infections differs from 29-90% [14] for skin and soft tissue infections to the mortality rate for bacteremia of 15-50 % [15].

This diverse cure rate/mortality rate can be partially ascribed to the increasing antibiotic resistance of *S. aureus* bacteria [16]. Shortly after the introduction of penicillin in 1940 into the clinic, the first penicillin resistant *S. aureus* (PRSA) strains were reported [17,18]. The same phenomenon occurred after the introduction of methicillin (MRSA) in 1960 [19]. As a consequence of emerging staphylococcal antibiotic resistance, the morbidity, mortality, length of hospitalization and costs are increasing. The costs of treatment can rise in some cases to more than $80,000 per patient [20]. The emerging resistance, in particular to the last-resort antibiotic vancomycin, is a serious public concern [16,21,22]. Therefore there is an urgent need for development of new anti-staphylococcal agents with novel mode of action.

*S. aureus* surface proteins

Cell wall-anchored (CWA) proteins are an interesting target for anti-staphylococcal therapy, as they are involved in a number of processes that are critical for the colonization and survival of *S. aureus*, by e.g. facilitating escape from the host immune system, promoting bacterial attachment to the extracellular matrix or host proteins and biofilm formation [23]. *S. aureus* CWA proteins, are exposed on the staphylococcal surface and are covalently anchored to the peptidoglycan of the staphylococcal cell wall by sortase A (SrtA) transpeptidase [24]. *S. aureus* SrtA mediates covalent anchoring of up to twenty four CWA proteins, exerting over 40 different functions, to the bacterial surface (Table 1) [23]. Therefore, targeting of *S. aureus* SrtA is interesting, as in this way all CWA proteins are targeted in one single step.
Sortases

Most Gram-positive bacteria express multiple sortase enzymes, with different structural features and biological functions [25-27]. Although these functions coincide in most species, there is an inter-species variation in primary sequence and substrate specificity (Table 2) [28,29]. Sortase enzymes process and covalently link a variety of functionally different CWA proteins to an amino group located on the peptide cross-bridge of peptidoglycan or polymerize proteins to build long filamentous polymers (pili). Both CWA proteins and pili, carry highly conserved specific cell wall sorting motifs that are recognized by sortases [25-27].

Based on the sequence homology, the sortase recognition motifs present in CWA proteins and the nucleophile used, sortases can be classified into six classes, of which classes A-D are most prevalent and have been studied most frequently (Table 2) [28,30,31].

*S. aureus* SrtA was discovered in 1999 by Schneewind and co-workers [24] after screening of 1000 mutants for a deficient capacity to anchor a fusion protein (resembling a native CWA protein structure) into the staphylococcal peptidoglycan. Two of the screened mutants accumulated the partially processed fusion protein within the bacterial cytoplasm and cell membrane, suggesting a defective sorting pathway. These defective *S. aureus* strains carried a mutation in the *surface protein sorting A* (*srtA*) gene [24]. The studies performed with the *srtA* deletion mutant strains revealed that SrtA transpeptidase is responsible for the anchoring of many virulence factors to staphylococcal cell wall, at least all those carrying the LPXTG SrtA recognition motif [32-35]. Strains lacking SrtA were not able to bind fibrinogen, fibronectin and IgG, suggesting that these strains possessed no functional ClfA/B, FnbpA/B and protein A surface proteins. The *srtA* KO strains seemed therefore to

### Table 1: Overview of *S. aureus* CWA proteins that are relevant for this thesis. For an extended description, see the review from Foster et al [23].

<table>
<thead>
<tr>
<th>Protein group</th>
<th>CWA protein</th>
<th>Ligand binding</th>
<th>Function</th>
<th>Role in infection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microbial surface component recognizing adhesive matrix molecules (MSCRAMMs)</td>
<td>Clumping factor A/B (ClfA/B), Fibronectin binding protein A/B (FnbpA/B)</td>
<td>Fibrinogen, Fibronectin</td>
<td>Adhesion to host proteins, extracellular matrix and cells</td>
<td>Endocarditis, Foreign body infection, Mastitis Abscess formation, Sepsis</td>
</tr>
<tr>
<td>Three-helical bundle motif protein A</td>
<td>Protein A</td>
<td>IgG Fc, IgM Fab VH3 subclass</td>
<td>Opsonophagocytosis inhibition, B cells superantigen</td>
<td>Pneumonia, Sepsis, Abscess formation</td>
</tr>
<tr>
<td>G5-E repeat family</td>
<td><em>S. aureus</em> surface protein G (SasG)</td>
<td>Unknown</td>
<td>Adhesion, Biofilm formation</td>
<td>Abscess formation</td>
</tr>
</tbody>
</table>
be attenuated in their virulence, as confirmed in several animal models (e.g. an increased survival of mice in septic arthritis and endocarditis models) [32-35]. It is therefore generally accepted that SrtA plays a key role in staphylococcal virulence.

In addition to *S. aureus*, srtA is found in a wide range of Gram-positive bacteria (Table 2), including the cariogenic bacterium *Streptococcus mutans* [36]. SrtA anchors the vast majority of CWA proteins to glycine residues of the peptidoglycan crossbridge peptide. However, some variations are known, for instance in internalins of *Listeria monocytogenes* the linkage occurs to the *m*-diaminopimelic acid crossbridge within the listerial peptidoglycan [37]. Surprisingly, recently the first sortase A-like enzyme has been found in a Gram-negative anaerobic bacteria, *Porphyromonas gingivalis*, associated with chronic periodontitis, an inflammatory disease of the supporting tissue of the teeth. The virulence factors, gingipains, are associated with the establishment of periodontitis. The gingipains are secreted or processed and attached to the cell surface of *P. gingivalis* in a sortase-A like dependent manner via an A-LPS [38].

The SrtB class is found in bacteria that also express SrtA, and is mostly involved in iron acquisition, such as in *S. aureus* [39]. The SrtC class is broadly distributed in Gram-positive

**Table 2**: Sortase classes together with their prevalence, function, substrates and nucleophiles.

<table>
<thead>
<tr>
<th>Sortase class</th>
<th>Bacteria</th>
<th>Function</th>
<th>Recognition motif</th>
<th>Nucleophile</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A C</td>
<td>Gram-positive bacteria</td>
<td>Anchoring of surface proteins Terminating of pilus assembly and anchoring of assembled pilus in Bacilli Pilus assembly in Corynebacteria</td>
<td>LPXTG</td>
<td>Lipid II Lys residues of pilins</td>
<td>[24,42-45]</td>
</tr>
<tr>
<td>B C</td>
<td>Staphylococci Streptococci Bacilli Clostridia Listeria</td>
<td>Anchoring of heme scavenging factors Pilus assembly in <em>S. pyogenes</em></td>
<td>N(P/A)(Q/K)(T/S) (N/G/S)(D/A) EVPTG</td>
<td>Peptidoglycan cross bridge Lys residues of pilins</td>
<td>[27,46-49]</td>
</tr>
<tr>
<td>D</td>
<td>Bacilli Clostridia</td>
<td>Attachment of proteins to facilitate sporulation</td>
<td>LPNTA</td>
<td>Peptidoglycan cross bridge</td>
<td>[50,51]</td>
</tr>
<tr>
<td>C</td>
<td>Streptococci Enterococci Bacilli Clostridium Actinomyces</td>
<td>Pilus assembly in Gram-positive bacteria</td>
<td>(I/L)(P/A)XTG</td>
<td>Lys residues of pilins</td>
<td>[28,52]</td>
</tr>
<tr>
<td>F</td>
<td>Corynebacteria Streptomyces</td>
<td>Unknown</td>
<td>divergent</td>
<td>unknown</td>
<td>[28,44]</td>
</tr>
<tr>
<td>E</td>
<td>Corynebacteria Streptomyces</td>
<td>Anchoring of assembled pilus in Corynebacteria Aerial hyphae formation</td>
<td>LAXTG</td>
<td>Lipid II</td>
<td>[28,41,45,53]</td>
</tr>
</tbody>
</table>
bacteria and is responsible for pili assembly [25], such as in oral pathogens *Streptococcus oralis* and *Actinomyces oris*. Here, the SrtC and SrtA classes are responsible for the pilus assembly and attachment, respectively. Pili on bacterial surface of these species mediate co-aggregation resulting in stable interspecies biofilms, which are associated with gingivitis and periodontitis [40]. SrtD class is responsible for covalent deposition of CWA proteins to sporulating bacteria, such as *Bacillus anthracis* (Table 2). The SrtD class and SrtD-dependent CWA proteins are expressed only during sporulation process. The mode of action of classes E and F still has to be elucidated [28,41] (Table 2). Until now, almost 5000 sortase genes have been identified in over 1700 species. Interestingly, sortase genes were also identified in two eukaryote species: the nematode *Caenorhabditis japonica* and the green algae *Volvox carteri* [31].

**S. aureus SrtA catalytic mechanism**

SrtA is a cysteine protease with a single conserved Cys184 in its active site, as the substitution of this cysteine for alanine [54] and inhibition with cysteine blocking agents (methanethiosulfonates and p-hydroxymercuribenzoic acid) [55] abolished the SrtA activity. SrtA is a single chain protein of 206 residues, in which two domains can be distinguished: an N-terminal stretch of hydrophobic amino acids (residues 1-59) embedded in the cytoplasmic membrane, which anchors the enzyme close to the bacterial surface and C-terminal catalytic domain (residues 60-206) [24]. NMR and X-ray crystallography revealed that the C-domain contains a unique eight stranded-β-barrel, which functions as a catalytic pocket, and a single α-helix that functions as a lid to close this pocket [56,57]. Within this pocket, the well-conserved His120 and Arg197 are positioned in close proximity to Cys184 and form a catalytic triad [58,59].

The SrtA catalytic mechanism has been studied extensively *in vitro* and *in vivo* [60,61]. *S. aureus* CWA proteins are synthesized in the cytoplasm as protein precursors. These precursors contain in addition to the N-terminal signal sequence, a highly conserved LPXTG SrtA recognition motif in the C-terminal part, followed by hydrophobic and positively charged regions [62,63]. Although in *S. aureus* glutamic acid is commonly found at position three [64], several studies have shown that SrtA processes LPMTG more efficiently [65,66]. In addition, the remaining leucine, proline, threonine and glycine residues are the only tolerated residues within the SrtA recognition motif [65]. Deletion experiments have shown that the complete C-terminal region is absolutely indispensable for incorporation of native CWA proteins [62]. CWA protein precursors are guided with their N-terminal signal sequence to the Sec-translocon located in cell membrane. After cleavage of the N-terminal signal sequence the CWA proteins remain associated with the membrane by the transmembrane hydrophobic segment and the positively charged segment [60]. SrtA cleaves the LPXTG motif between threonine and glycine, resulting in the formation of an acyl enzyme intermediates between the enzyme and the substrate [54,67]. In the subsequent transpeptidation
reaction the acyl intermediate is covalently anchored to an amino group of the pentaglycine crossbridge of lipid II [43,68,69]. In vitro data show that SrtA is highly selective for glycine with diglycine to be the minimal unit for successful transpeptidation reaction [70]. The CWA-lipid II complex functions as a substrate for penicillin binding proteins (PBP) and is incorporated via transpeptidation and transglycosylation reactions into the mature peptidoglycan [71,72]. CWA protein recognition, processing and anchoring by endogenous S. aureus SrtA is summarized in Figure 1. The most likely mechanism of SrtA is the reverse protonation reaction, proposed by Frankel and co-workers, as shown in Figure 2 [73].

**Figure 1:** Processing of S. aureus CWA proteins by SrtA.

A: The synthesis of CWA proteins takes place in the cytoplasm by ribosomes. B: The CWA protein precursor contains a signal peptide (Sp) on the N-terminus for specific targeting and a C-terminal sorting signal with a highly conserved LPXTG recognition motif, followed by 15-22 hydrophobic amino acids and by 5-12 mostly positively charged residues [62,63]. C: CWA proteins are translocated via the general secretory (Sec) translocon, there the signal sequence is removed by the signal peptidase (SPase) [74]. D: After secretion, the peptide precursor can then be retained in the plasma membrane by means of hydrophobic amino acids and by positively charged residues. E: Subsequently, the peptide precursor can be recognized by means of the LPXTG motif by SrtA, a transpeptidase which catalyzes the cleavage between the threonine residue and the glycine residue within the recognition motif [67]. Catalysis is initiated when the thiol group of amino acid C184 in the SrtA active site nucleophilically attacks the carbonyl carbon or the threonine residue. F: The resulting free carboxyl group of threonine reacts with the C184 thiol group in the SrtA active site which leads to the formation of thioacyl-linked protein-sortase intermediate [54]. After the cleavage, the transpeptidation reaction follows. G: In this step, SrtA joins the carboxyl group of threonine to the free amino group of the pentaglycine, the cell wall precursor lipid II, resulting in the formation of an amide bond [69]. H: This lipid II linked protein complex is then used as a substrate for the transglycosylation and transpeptidation reactions. In this way, S. aureus SrtA covalently anchors all CWA proteins bearing the LPXTG pentapeptide recognition motif [23].
SrtA inhibitors

SrtA plays an important role in the pathogenesis of S. aureus and is therefore a potential target for novel therapeutics. Screening of libraries of both natural (Table 3) and synthetic compounds has identified a large number of SrtA inhibitors [75-78]. Purification of one of the plant extracts (from Fritillaria verticillata) led to the isolation of β-sitosterol-3-O-glucopyranoside against S. aureus [76]. Morin, belonging to flavonols derived from Chinese herbs and fruit, is another SrtA natural inhibitor against S. aureus and S. mutans. Morin was found to reduce the fibrinogen dependent S. aureus clumping [79] and biofilm formation in S. mutans [80]. In addition, biofilm formation of S. mutans can be inhibited in a SrtA-dependent manner by curcumin, a compound isolated from Curcuma longa L. rhizome [81]. In S. aureus, curcumin inhibits the fibronectin binding in a dose-dependent manner (2.5-20 µg/ml) [82]. Higher fibronectin inhibiting capacity (2-16 µg/ml) in S. aureus was found with isoaaptamine, isolated from marine sponge the Aaptos aaptos [83].

Despite the large number of available SrtA inhibitors [84,85], in vivo data are still limited. This might be due to the fact that lots of these inhibitors lack specificity, are toxic and/or have a low activity that make their use as a therapeutic not desirable. The synthetic SrtA inhibitor (Z)-3-(2,5-dimethoxyphenyl)-2-(4-methoxyphenyl)acrylonitrile (DMMA) was the first SrtA inhibitor that has been tested in a mouse model [86]. DMMA treatment resulted in an increased dose-dependent survival rates with IC₅₀ of 9.2 µM and CFU decrease in kidney and bones. Infection of mice with a srtA KO S. aureus strain resulted in significantly
lower survival rates in comparison to DMMA treated mice infected with WT *S. aureus*. Survival of mice infected with *srtA/B* *S. aureus* KO strain was not significantly different from that of mice infected with the WT *S. aureus* strain, that were treated with DMMA. These data suggest that DMMA inhibits both SrtA and SrtB [86]. In general, treatment with a SrtA inhibitor leads to attenuation of the virulence, but not to bacterial killing. Since *S. aureus* infections predominantly occur in immune compromised hosts, killing of bacteria is more desirable than attenuation of their virulence.

Table 3: Selection of representative SrtA (*S. aureus* and *S. mutans*) inhibitors from natural origin.

<table>
<thead>
<tr>
<th>Name</th>
<th>Source</th>
<th>SrtA IC50 (µg/ml)</th>
<th>Effect</th>
<th>MIC (µg/ml)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-sitosterol-3-O-glucopyranoside</td>
<td><em>E. verticillata</em></td>
<td>18.3 (<em>S. aureus</em>)</td>
<td>Not determined</td>
<td>200 (<em>S. aureus</em>)</td>
<td>[76]</td>
</tr>
<tr>
<td>Morin</td>
<td>Chinese herbs and fruit</td>
<td>11.29 (<em>S. aureus</em>)</td>
<td>Fibrinogen binding inhibition (<em>S. aureus</em>)</td>
<td>&gt; 900 (<em>S. aureus</em>)</td>
<td>[79,80]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8.21 (<em>S. mutans</em>)</td>
<td>Biofilm formation inhibition (<em>S. mutans</em>)</td>
<td>No effect on viability (<em>S. mutans</em>);</td>
<td></td>
</tr>
<tr>
<td>Curcumin</td>
<td><em>C. longa</em> L.</td>
<td>13.8 (<em>S. aureus</em>)</td>
<td>Fibrinectin binding inhibition (<em>S. aureus</em>)</td>
<td>123.5 (<em>S. aureus</em>);</td>
<td>[81,82,87]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3.6 (<em>S. mutans</em>)</td>
<td>Biofilm formation inhibition (<em>S. mutans</em>)</td>
<td>61.7 (<em>S. mutans</em>);</td>
<td></td>
</tr>
<tr>
<td>Isoaaptamine</td>
<td><em>A. aaptos</em></td>
<td>3.7 (<em>S. aureus</em>)</td>
<td>Fibrinectin binding inhibition (<em>S. aureus</em>)</td>
<td>50 (<em>S. aureus</em>);</td>
<td>[83]</td>
</tr>
</tbody>
</table>
AIM AND OUTLINE OF THIS THESIS

In this thesis the feasibility of SrtA as a target for anti-infective therapy has been examined. In contrast to conventional approaches seeking to fight S. aureus by inhibiting SrtA, it was explored if endogenous S. aureus SrtA could be exploited to chemically modify in situ the cell wall of S. aureus to enable fundamental and clinical applications.

In Chapter 2, the development of a novel multiplex assay for the rapid yet comprehensive characterization of CWA proteins and carbohydrates on surface of various S. aureus strains in different growth phases and growth conditions is described. In Chapter 3, the performance of non-magnetic beads and the newly developed magnetic beads in antibody detection directed against S. aureus and S. pneumoniae antigens is compared. Chapter 4 is a review of the background of S. aureus SrtA, focusing on its application in the preparation of cyclic proteins and peptides. In Chapter 5, the generation of SrtA specific fluorescent synthetic substrates equipped with an LPETG SrtA recognition motif is described and its incorporation is characterized in different growth phases. Since relatively high concentrations (mM) of the LPETG substrate were required for incorporation, in Chapter 6 was aimed to improve the incorporation efficiency by elongation of the LPETG core motif with various membrane or lipid II binding moieties. In Chapter 7, the role of lipid II was examined in the incorporation of synthetic SrtA substrates in the cell wall using various lipid II targeting antibiotics. Chapter 8 provides the reader with a summary and general discussion of the results, described in this thesis.
REFERENCES