Chapter 7

Covalent incorporation of synthetic sortase A substrates into the Staphylococcus aureus cell wall proceeds via a lipid II-independent pathway

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ABSTRACT

*Staphylococcus aureus* endogenous sortase A (SrtA) covalently incorporates cell wall anchored proteins equipped with a SrtA recognition motif (LPXTG) via a lipid II-dependent pathway into the staphylococcal peptidoglycan layer. Previously, we found that the endogenous *S. aureus* SrtA is able to recognize and process a variety of exogenously added synthetic SrtA substrates, such as K(FITC)LPMTG-amide and K(FITC)-K-vancomycin-LPMTG-amide, and to incorporate them covalently, in a SrtA-dependent manner and with different efficiencies, into the bacterial peptidoglycan. In this study, we studied if native and synthetic substrates are processed by SrtA via the same pathway. First, the effect of a synthetic substrate (LPMTG-amide) was investigated on the incorporation and surface localization of protein A, a native SrtA substrate. Secondly, the effect of lipid II inhibiting antibiotics was assessed on the incorporation of native and synthetic SrtA substrates. This revealed that in bacteria cultured in the presence of LPMTG-amide neither the amount of incorporated protein A nor its localization were affected in a specific way. Treatment of *S. aureus* with bacitracin, an inhibitor of lipid II, resulted in a decreased incorporation of protein A in the bacterial cell wall, whereas that of exogenous synthetic substrates was increased. These results support that physiological and exogenous synthetic substrates are processed by SrtA via different pathways.
INTRODUCTION

*Staphylococcus aureus* (*S. aureus*) expresses a large number of virulence factors, which play a key role in the successful establishment of an infection [1,2]. Among these are a variety of proteins that are covalently linked to the peptidoglycan (PG) layer of the bacterium, designated cell wall anchored (CWA) proteins. CWA proteins have been implicated in a variety of processes deemed to be important for the successful colonization and infection of *S. aureus*, such as adhesion to host tissues, invasion in epithelial cells, evasion of the host’s immune system and biofilm formation [1]. The covalent anchoring of these factors into the staphylococcal cell wall is catalyzed by sortase A (SrtA), a membrane associated enzyme [3]. CWA proteins of *S. aureus* share a common architecture in their C-terminal region encompassing three domains: (i) the SrtA recognition motif LPXTG, Leu-Pro-X-Thr-Gly (X represents any possible amino acid); (ii) a hydrophobic transmembrane domain and (iii) a tail of positively charged amino acids [4,5]. The last two domains sequester the CWA proteins to the plasma membrane prior to recognition of the LPXTG motif by SrtA. SrtA cleaves LPXTG between Thr and Gly [6], resulting in the formation of an acyl-enzyme intermediate. This intermediate is resolved by a nucleophilic attack by the amino group of the pentaglycine side chain of lipid II (undecaprenol-pyrophosphoryl-MurNAc(GlcNAc)-Ala-D-isoGlu-Lys(ε-Gly3)-D-Ala-D-Ala) [7-9]. Then, a transglycosylation reaction follows, in which the sugar subunits (MurNAc-GlcNAc) of lipid II within the CWA-lipid II complexes are polymerized with neighboring sugar subunits of other lipid II and/or CWA-lipid II complexes to generate PG strands [10,11]. Subsequently, the PG strands are cross-linked in a transpeptidation reaction, in which a Penicillin Binding Protein (PBP) enzymatically cleaves the bond between the terminal D-Ala D-Ala of lipid II or CWA-lipid II complexes, with concomitant formation of an amide bond with an accessible pentaglycine of a neighboring strand leading to the stable PG structure [11]. In this way, CWA proteins are incorporated into the growing PG in a lipid II-dependent manner and ultimately exposed in the mature staphylococcal PG [8,9,12,13].

The CWA protein incorporation can be inhibited with peptidoglycan synthesis inhibiting antibiotics [8,14]. These include: (i) beta-lactams like penicillin G which mimics the D-Ala D-Ala motif and thereby inhibits the PBP catalyzed transpeptidation reaction [15]; (ii) Vancomycin, which blocks transglycosylation and transpeptidation reactions by its specific binding to the accessible D-Ala D-Ala terminus and its bulky structure [16]; (iii) Bacitracin which selectively binds and sequesters the C55-pyrophosphate species formed and recycled during lipid II synthesis [17]; (iv) and the lantibiotic nisin which recognizes the pyrophosphate moiety of lipid II by its N-terminal region, which contains A and B lantionine rings [18], C-terminus (containing C, D and E rings) is responsible for subsequent pore formation) [19,20].
In previous studies it was found that SrtA also incorporates exogenously added synthetic SrtA substrates equipped with the SrtA recognition motif [21-23]. However, little if any competition occurred with physiological substrates. Moreover, while incorporation of physiological substrates peaked in the logarithmic growth phase, highest incorporation of synthetic substrates occurred in the stationary phase [21]. Aim of the present study was to elucidate the cause of the different behavior of physiological and synthetic SrtA substrates. To do so, the possible role of lipid II in incorporation of protein A, a physiological SrtA substrate was compared with that of K(FITC)LPMTG-amide and K(FITC)-K-vancomycin-LPMTG-amide, synthetic substrates [22].

MATERIALS AND METHODS

Peptide synthesis and procedure
FITC-labelled and non-labelled peptide synthesis, purification and confirmation of authenticity were performed as previously described [21-25]. The substrates numbered 1-5 used in this study are shown in Table 1. The nisin A/B azide species was prepared as previously described [26]. The unique carboxyl groups of nisin A/B were labelled with 5-(aminoacetamido)fluorescein(AAFlu) as described for nisin by Haser et al [27].

Table 1: The amino acid sequences of SrtA synthetic substrates used in this study.

<table>
<thead>
<tr>
<th>Substrate number</th>
<th>N-term</th>
<th>Substrate sequence</th>
<th>C-term</th>
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<tbody>
<tr>
<td>1</td>
<td></td>
<td>LPMTG</td>
<td>amide</td>
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<tr>
<td>2</td>
<td></td>
<td>GTMPL</td>
<td>amide</td>
</tr>
<tr>
<td>3</td>
<td>K(FITC)</td>
<td>LPMTG</td>
<td>amide</td>
</tr>
<tr>
<td>4</td>
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<td>K(Vancomycin)LPMTG</td>
<td>amide</td>
</tr>
<tr>
<td>5</td>
<td>K(FITC)</td>
<td>K(Vancomycin)MGTL</td>
<td>amide</td>
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Bacterial strains
S. aureus 8325-4 wild type (WT) strain together with its isogenic srtA deletion mutant (srtA KO) and protein A deletion mutant (spa KO) were used. The srtA mutant was generated by srtA:ermC allele transduction, as previously described; mutants were selected and maintained on erythromycin plates (3 µg/ml) [21,28]. The spa KO strain was maintained on tetracycline plates (3 µg/ml).

Incorporation and localisation of protein A in the presence of exogenous substrates
WT, srtA KO and spa KO strains were grown overnight on brain-heart infusion agar (BHA, BD (Becton Dickinson and Company)-Difco, Etten-Leur, The Netherlands), BHA supplemented with 3 µg/ml erythromycin (Abbott Laboratories, U.S.) and BHA supplemented with
3 µg/ml tetracycline (Sigma-Aldrich, Zwijndrecht, The Netherlands), respectively. Then the bacteria were suspended in Luria-Bertani (LB) medium (BD-Difco) to obtain OD_{600nm} 0.1. Twenty-five microliters of *S. aureus* bacterial suspensions were supplemented with either 25 µl of i) LB medium; ii) 2 mM; 1 mM or 0.5 mM of LPMTG-amide substrate or iii) 2 mM; 1 mM or 0.5 mM of GTMPL-amide substrate (used as a control) in 96 wells-plates (all substrates were diluted in LB medium). The bacteria were cultured until exponential growth phase (OD_{600nm} 0.5) was reached in the dark at 37 °C with continuous shaking (450 rpm, thermomixer plate shaker, Eppendorf). Then 3 PBS washes, 1 wash with 1 % sodium dodecyl sulphate (SDS) (Merck-Schuchardt OHG, Germany) in PBS at 60 °C for 5 min to remove the non-covalently bound and intracellular substrates and 3 additional PBS washes followed. The individual bacterial pellets were suspended in 10 µl SrtA buffer (50 mM Tris, 150 mM NaCl, 10 mM CaCl₂, pH 7.5) and incubated with 10 µl of 1:50 dilution anti-protein A IgY-FITC (FITC-conjugated Chicken anti-Protein A, Gallus Immunotech Inc., Fergus, Canada) for 45 min with continuous shaking (800 rpm, thermomixer plate shaker, Eppendorf) at ambient temperature in the dark. After the incubation with a fluorescent label the bacteria were washed 3 times with PBS and their fluorescence was quantified and qualified with an Accuri C6 Flow Cytometer (BD Biosciences) and with a phase contrast fluorescence microscope (Olympus IX51, Leiden, the Netherlands), respectively.

**Effect of PG synthesis inhibitors on protein A incorporation**

Cell wall synthesis inhibition during bacterial growth was performed as previously described with several modifications [8]: WT and srtA KO strains were grown as described above. Subsequently, bacteria were suspended in LB medium to obtain an OD_{600nm} of 0.05 in a volume of 4 ml. The bacteria were cultured for 30 min at 37 °C with continuous shaking (230 rpm). Then either 10 µg/ml penicillin, 10 µg/ml vancomycin or 20 µg/ml bacitracin (Sigma-Aldrich) were added to individual bacterial cultures and the incubation proceeded at 37 °C under continuous shaking (230 rpm) until the untreated bacteria had reached an OD_{600} of 0.5. The OD_{600} was recorded every 30 min. After incubation, the bacterial densities were normalized based on OD_{600}. The bacteria were washed twice with PBS followed by centrifugation for 5 min at 3,700 x g. The individual bacterial pellets were suspended in 100 µl SrtA buffer and 10 µl of individual WT or srtA KO bacterial cultures were incubated with 10 µl of 1:50 dilution anti-protein A IgY in SrtA buffer during 45 min in the dark at ambient temperature with continuous shaking (800 rpm).

In addition, the effect of these antibiotics was examined in non-growing bacteria. In brief, WT and srtA KO bacteria were cultured until the exponential growth phase OD_{600} of 0.5 at 37 °C with continuous shaking (230 rpm). After incubation, bacteria were washed twice with PBS followed by centrifugation for 5 min at 3,700 x g. The pellet was suspended in 100 µl SrtA buffer and either 10 µg/ml penicillin, 10 µg/ml vancomycin, 20 µg/ml bacitracin antibiotics or 0, 1, 10, 25, 50 or 200 µg/ml of nisin A/B domain were added and the individual
cultures were incubated for 45 min at ambient temperature with continuous shaking (150 rpm). After incubation with antibiotics, the individual WT or srtA KO bacterial cultures were incubated with anti-protein A IgY in SrtA buffer. Bacteria exposed to the nisin A/B domain were incubated with 1 mM of K(FITC)LPMTG-amide overnight in 50 µl (25 µl bacterial culture in SrtA buffer and 25 µl substrate dissolved in SrtA buffer). After incubation bacteria were washed three times with PBS followed by centrifugation for 5 min at 3,700 x g. In addition, bacteria were treated with 1 % SDS at 60 °C for 5 min to remove the non-covalently bound and intracellular substrate and 3 additional PBS washes followed. The stained bacteria were measured with the Accuri C6 Flow Cytometer and analysed using Accuri C6 software (BD Biosciences).

**Short treatment of S. aureus with bacitracin**

The WT and srtA KO bacteria were cultured in a volume of 4 ml until OD$_{600nm}$ of 0.400 was reached, as described above. One tube (zero time-point) was stored on ice until measurement. To other tubes, either LB medium or 1 mg/ml bacitracin (50 µl per tube) was added and incubation proceeded for 15 or 45 min. Then, bacteria were collected by centrifugation (5 min at 3,700 x g) and suspended in PBS to OD$_{600nm}$ of 0.400. The bacteria were washed twice with PBS followed by centrifugation for 5 min at 3,700 x g. One half of the individual bacterial pellets was suspended in 50 µl PBS and the other half was suspended in SrtA buffer. Ten microliter of individual bacterial pellet per strain per condition suspended in PBS was incubated with either 10 µl of 200 µg/ml FITC-labelled nisin A/B domain, 10 µl of 1 µg/ml vancomycin-BODIPY (4,4-difluoro-4-bora-3a,4a-diaza-s-indacene) conjugate (Invitrogen) or 10 µl of 1:50 dilution anti-protein A IgY for 45 min in the round bottom plates in the dark at ambient temperature with continuous shaking (800 rpm). In parallel, 25 µl of individual bacterial pellets per strain per condition suspended in SrtA buffer were incubated with 25 µl of either K(FITC)LPMTG-amide substrate (end concentration of 1mM), K(FITC)-K-vancomycin-LPMTG-amide or K(FITC)-K-vancomycin-MGTLP-amide substrates (both end concentration of 5 µM) in a flat bottom plates for 17 hrs in the dark at 37 °C with continuous shaking (220 rpm). After the incubation bacteria were washed three times with PBS followed by centrifugation for 5 min at 3,700 x g. In addition, bacteria incubated with the synthetic SrtA substrates were treated with 1 % SDS at 60 °C as described above. The stained bacteria were suspended in 200 µl PBS and analysed on the Accuri C6 Flow Cytometer with Accuri C6 software.

**Statistical analysis**

Statistical analysis was performed using the Prism 5.0 package (GraphPad software, San Diego, CA, USA). We used Kruskal Wallis test with Dunn's posttest and one-way ANOVA testing with Bonferroni correction, considering P < 0.05 as being statistically significant. All
experiments were independently repeated three times and the mean with Standard Error on the Mean (SEM) or the median with range were depicted were applicable.

RESULTS AND DISCUSSION

Effect of LPMTG-amide on incorporation and localization of protein A
In our previous study, we found that K(FITC)LPMTG-amide, a synthetic SrtA substrate, is covalently incorporated into the staphylococcal cell wall more efficiently than the parent substrate K(FITC)LPETG-amide [22]. Here, the competition of LPMTG-amide substrate on endogenous S. aureus substrate was examined. We assessed the effect of synthetic SrtA substrates (LPMTG-amide and GTMPL-amide, scrambled control) on incorporation and localisation of protein A with anti-protein A IgY. The incubation of S. aureus in the presence of an increasing concentration of LPMTG-amide SrtA specific substrate resulted in a dose dependent reduction of protein A incorporation. A comparable effect, however, was observed after incubating the bacteria in the presence of GTMPL-amide, the scrambled version of the substrate, which is not processed by SrtA. This indicates that the decrease in protein A incorporation was not caused by a specific inhibitory effect of LPMTG-amide. The srtA KO strain expressed little if any covalently bound protein A, confirming the involvement of SrtA in anchoring of this CWA protein (Figure 1A).

In addition, protein A appears to be distributed in a ring-like structure on the bacterial cell wall of WT S. aureus strain and the incubation with the LPMTG-amide substrate did not alter this distribution. The spa KO and srtA KO strains did not reveal any fluorescence (Figure 1B). These results are in line with previous studies showing that synthetic SrtA substrates do not exert inhibitory effects on the incorporation of protein A [21,23].

The effect of lipid II inhibitors on the incorporation of protein A and synthetic SrtA substrates

Previous studies showed that exogenous, synthetic SrtA substrates are covalently anchored into the bacterial cell wall by endogenous SrtA [21,22]. These studies, however, left the role of lipid II (the primary acceptor for CWA proteins) in this process unresolved [8,9,14]. To study the role of lipid II in incorporation of native and synthetic SrtA substrates, the effect of several antibiotics targeting different stages of the PG synthesis were tested for their effect on SrtA-mediated incorporation of protein A, K(FITC)LPMTG-amide and K(FITC)-K-vancomycin-LPMTG-amide. These included penicillin G, vancomycin and bacitracin. First, S. aureus was cultured for 30 minutes in the absence of antibiotics. Then, the antibiotics were added and culturing was continued for another 2 hours after which the cells were harvested and their protein A incorporation was evaluated using anti-protein A IgY. The srtA KO strain was used as negative control. After 60 minutes in the presence of either penicillin
G or vancomycin OD₆₀₀ did not increase anymore in both tested strains, in contrast to the untreated control (Figure 2A-B; P < 0.0001). Interestingly, bacitracin did inhibit the growth of S. aureus WT strain more effectively than that of the srtA KO stain (Figure 2A-B; P < 0.0001 vs. P < 0.01). FACS analysis revealed that penicillin and bacitracin resulted in a more than 50 % reduction of protein A anchoring on bacterial surface. Vancomycin treatment resulted in a moderate reduction of protein A display (Figure 2C). To rule out a direct effect of antibiotics on the protein A measurement by FACS, S. aureus was incubated with antibiotics under non-growing conditions for 45 minutes. FACS analysis revealed no effects on protein A display (Figure 2D).

We observed that prolonged culturing of S. aureus in the presence of antibiotics made the bacteria fragile, causing a large variation in the results of the FACS analysis. Furthermore, it is conceivable that bacteria that have grown under stressful conditions may have undergone
Role of Lipid II in the Incorporation of Substrates

The role of lipid II in the incorporation of substrates is complicated by various phenotypical changes. This makes unambiguous interpretation of the results difficult. We, therefore, decided to treat the bacteria for relatively short periods of time (15 and 45 minutes) with bacitracin. These short term bacitracin pulses had no effect on bacterial growth, as monitored by OD 600 measurements (data not shown). The effect of bacitracin on the expression of lipid II was measured by incubating the bacteria afterwards with the FITC-nisin A/B domain or BODIPY-labelled vancomycin, binding to the pyrophosphate linkage group and the D-Ala-D-Ala moiety of lipid II, respectively. Bacitracin pulses resulted in approximately 30% reduction of FITC-nisin A/B domain binding to the WT and the srtA KO strains compared to the LB- and untreated controls (Figure 3A). Only a moderate decrease in vancomycin-BODIPY binding was measured in both S. aureus strains (Figure 3B). This suggests that vancomycin in addition to D-Ala-D-Ala motifs on lipid II, also binds to D-Ala D-Ala motifs present in the peptidoglycan layer (prior to their cleavage in the transpeptidation step). These data are in line with a previous study performed on Bacillus subtilis, where it was found that vancomycin binds to the externalized membrane-bound unincorporated lipid II molecules as well as to free D-Ala D-Ala within the peptidoglycan layer [29]. FACS

![Figure 2: Protein A incorporation after PG synthesis inhibition.](image-url)

Growth curves of (A) WT and (B) srtA KO S. aureus bacteria are shown upon addition of either 10 µg/ml penicillin, 10 µg/ml vancomycin or 20 µg/ml bacitracin after 30 min of incubation (depicted with an arrow). The bacterial growth was measured every 30 min at OD 600 (x-axis) until the untreated bacteria reached OD 600 of 0.5 (y-axis). Then the bacteria were incubated with (C) anti-protein A IgY (FITC-labelled) to determine the protein A incorporation. The median fluorescence was determined by FACS (y-axis). To determine the influence of antibiotic in static bacteria, S. aureus WT and srtA KO were grown in the absence of the antibiotics until the exponential growth phase was reached and the antibiotics were added in SrtA buffer. Then, the bacteria were incubated with (D) anti-protein A IgY and FACS analysis followed (median fluorescence is depicted on the y-axis). The significant differences are marked with asterisks (*P < 0.05; *** P < 0.0001). We used one-way ANOVA testing with Bonferroni correction and Kruskal Wallis met Dunn’s posttest for figure 2A-B and 2C, respectively.
analysis revealed an approximately 1.5-fold increase in the protein A content in untreated bacteria between 15 and 45 minutes of culturing (Figure 3C). In the presence of bacitracin, however, protein A was no longer incorporated in this time interval (Figure 3C). These results support previous studies showing that protein A is anchored in the peptidoglycan layer via a lipid II-dependent pathway [8].

In parallel, the effect of bacitracin treatment was tested on incorporation of K(FITC) LPMTG-amide and K(FITC)-K-vancomycin-LPMTG-amide. In this case, enhanced incorporation of synthetic SrtA substrates occurred in bacitracin-treated bacteria (Figure 4A-B). After 15 and 45 minutes of growth in the presence of bacitracin, the incorporation of K(FITC)LPMTG-amide was higher by 30 % and 50 %, respectively, compared to that in untreated bacteria (Figure 4A). With K(FITC)-K-vancomycin-LPMTG-amide even higher incorporation was found being 30 % and 70 % higher than the controls after treatment for 15 or 45 minutes (Figure 4B). No incorporation was found for the scrambled substrate (K(FITC)-K-vancomycin-MGTLP-amide), nor for the native substrates (K(FITC)LPMTG-
amide and K(FITC)-K-vancomycin-LPMTG-amide) in the srtA KO strain, confirming the specificity of the incorporation which is solely dependent on staphylococcal endogenous SrtA transpeptidase (Figure 4A-B). The increased incorporation of K(FITC)-K-vancomycin-LPMTG-amide supports that vancomycin binds to D-Ala D-Ala motifs present in the mature PG layer, as shown in Figure 3B. Furthermore, the increased incorporation of specific SrtA synthetic substrates upon inhibition of lipid II, strongly suggests that lipid II does not play a role in the incorporation of these substrates. We additionally examined this by testing the effect of treatment with nisin A/B domain, which binds to the pyrophosphate moiety of lipid II, on the incorporation of K(FITC)LPMTG-amide. This revealed that nisin A/B domain in concentrations up to 200 µg/ml, had no effect on incorporation of this substrate (Figure 4C). This further corroborates that these exogenous SrtA substrates are directly covalently linked to free pentaglycines within the mature bacterial cell wall, without intermediate binding to lipid II. The increase of accessible free pentaglycine units within the staphylococcal PG can be explained by the decreased availability of CWA-lipid II adducts after bacitracin treatment, that are covalently linked by PBPs to free pentaglycines.

Figure 4: The detection of SrtA synthetic substrate incorporation after lipid II inhibition with bacitracin and nisin A/B domain. (A-B) WT and srtA KO S. aureus strains (depicted on the x-axis) were cultured in the presence of either LB medium or bacitracin, as depicted next to the figures and as described in the legend of figure 3 more in detail. Then, the bacteria were incubated with (A) either 1 mM of substrate 3 (=K(FITC)LPMTG-amide) or (B) 5 µM of substrates 4 (=K(FITC)-K-vancomycin-LPMTG-amide) and 5 (=K(FITC)-K-vancomycin-MGTLP-amide) in SrtA buffer. (C) WT and srtA KO S. aureus bacteria were incubated with increasing concentrations of nisin A/B domain (0-200 µg/ml, depicted on the x-axis) in SrtA buffer. Then, bacteria were incubated with 1 mM of substrate 3 (=K(FITC)LPMTG-amide) and median fluorescence was determined by FACS scan, as depicted on the y-axis. Kruskal Wallis met Dunn's posttest was used as a statistical test.
within mature PG via a transpeptidation reaction [30]. This hypothesis is supported by the data presented in Figure 3C and 4A-B, where we show that decreased protein A display on bacterial surface is complementary to the increased SrtA substrate incorporation after bacitracin treatment. In addition, these data suggest that the SrtA transpeptidase is also able to utilize the available pentaglycine units within the mature peptidoglycan for incorporation of exogenous synthetic SrtA substrates.

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