Equilibrium of sortase A dimerization on *Staphylococcus aureus* cell surface mediates its cell wall sorting activity

Jie Zhu¹, Liang Xiang^{2,4}, Faqin Jiang^{1,3} and Zhiwen J Zhang^{1,4}

¹Division of Medicinal Chemistry, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712, USA; ²Department of Biomedical Engineering, The University of Texas at Austin, Austin, TX 78712, USA; ³Department of Medicinal Chemistry, School of Pharmacy, Shanghai Jiao Tong University, Shanghai 200240, China; ⁴Bioengineering Department, School of Engineering, Santa Clara University, Santa Clara, CA 95053, USA

Corresponding author: Zhiwen J Zhang. Email: zzhang@scu.edu

Abstract

Staphylococcus aureus sortase A (SrtA) transpeptidase is a therapeutically important membrane-bound enzyme in Gram-positive bacteria, which organizes the covalently attached cell surface proteins on the peptidoglycan cell wall of the organism. Here, we report the direct observation of the highly selective homo-dimerization of SrtA on the cell membrane. To address the biological significance of the dimerization towards enzyme function, site-directed mutagenesis was performed to generate a SrtA mutant, which exists as monomer on the cell membrane. We observed that the cell surface display of adhesive proteins in *S. aureus* cells expressing monomeric SrtA mutant is more prominent than the cells expressing the wild-type enzyme. A cell-based invasion assay was also performed to evaluate the activities of wild-type SrtA and its monomeric mutant as well. Our data demonstrated that *S. aureus* cells expressing SrtA in monomeric form invade host mammalian cells more efficiently than those expressing wild-type SrtA in dimer-monomer equilibrium. The results suggested that the monomeric form of SrtA is more active than the dimeric form of the enzyme in terms of cell surface display of virulence factors for infection. This is the first study to present the oligomerization of SrtA and its related biological function on the cell membrane. Study of SrtA dimerization has implications for understanding its catalytic mechanism at the cellular level as well as the development of novel anti-infective agents.

Keywords: Sortase A, dimerization, cell sorting activity, cell membrane

Experimental Biology and Medicine 2016; 241: 90–100. DOI: 10.1177/1535370215592122

Introduction

Staphylococcus aureus (*S. aureus*) is the primary cause of hospital-acquired infections today.¹ The alarming prevalence of multidrug-resistant *S. aureus* (MRSA) strains over the past decade has made it imperative to identify novel and effective drugs to treat these infections.² Sortase A (SrtA) is commonly used in Gram-positive bacteria including *S. aureus* for displaying many cell surface proteins as virulence factors that are necessary for bacterial infections and formation of biofilms.^{3–6} SrtA mutants exhibit severe defects in virulence, which suggests that it is a good drug target for the development of new type of anti-infective agents.⁷

S. aureus SrtA is a 206 amino acid membrane protein consisting of a N-terminal transmembrane domain, a linker region and an extra-cellular C-terminal catalytic domain.⁸ SrtA is a cysteine transpeptidase, recognizing its first substrate with a LPXTG sequence (X can be any amino acid)⁹ and subsequently cleaving between the T and G to form a thioester-linked acyl-enzyme intermediate. Next, the

terminal amino group from the second substrate penta-glycine on the cell wall cross-bridge nucleophilically attacks the thioester intermediate to form a covalent linkage between the first substrate to peptidoglycan cell wall.^{8,10–12} NMR and X-ray crystallography structural data of the C-terminal catalytic domain of *S. aureus* SrtA have revealed that the enzyme folds into a unique eight-stranded β -barrel with several helices and loops.^{13,14} More than a dozen small molecule inhibitors targeting SrtA active site have been developed but not yet been proved with therapeutic efficacy *in vivo*.⁷ Recently, it has been suggested that the biological function of sortase family proteins are regulated *in vivo* at different levels, such as transcription,¹⁵ and sub-cellular localization.^{16,17} Therefore, a comprehensive understanding of SrtA mechanism is necessary for developing effective SrtA inhibitors that can function *in vivo*. Recent studies have demonstrated that the C-terminal catalytic domain of SrtA selectively forms a homo-dimer *in vitro* and in *E. coli*.^{18–21} Herein, we demonstrate that SrtA also forms homo-dimer on the membrane of *S. aureus* and such

dimerization mediates SrtA's activities on the cell membrane, suggesting that the dimerization of SrtA is one of the mechanisms to globally coordinate the cell surface decoration on *S. aureus*.

Materials and methods

Construction of SrtA knockout *S. aureus* strain

A SrtA knockout strain (*srtA*[−]) served as the host for assays was generated by using the TargeTron™ Gene Knockout System (Sigma-Aldrich, St. Louis, MO). TargeTron™ Gene Knockout System uses site-specific group II intron insertions to permanently disrupt genes on the bacterial genomic DNA.²² A web-based algorithm was used to locate an optimal intron insertion site between the 93rd and 94th nucleotide in SrtA. This gave a score of 7.55 and E-value as 0.152. The primers designated as IBS (5'-AAAAAGCTTAT AATTATCCTTACATATCGATAATGTGCGCCAGATAG-GGTG-3'), EBS1d (5'-CAGATTGTACAAATGTGGT GATAACAGATAAGTCGATAATTATAACTTACCTTTCT-TTGT-3'), EBS2 (5'-TGAACGCAAGTTTCTAATTC GGTATATGTCGATAGAGGAAAGTGTCT-3') and EBS universal were used to perform the PCR reaction and amplify a 350 bp fragment from the template pNL9164. The amplified 350 bp fragment, which targets the intron to the SrtA sequence, was digested with Hind III and BsrG I (New England Biolabs, Beverly, MA) and inserted into pNL9164 to make pNL9164-srtA. The plasmid pNL9164-srtA was then transformed into *S. aureus* strain RN4220 (general gift from Dr. Alan Lambowitz). These cells were grown in tryptic soy broth (TSB) at 32 °C for one hour and diluted at a ratio of 1:5 into fresh media containing 10 µg/mL erythromycin (Sigma, St. Louis, MO). Afterwards, the overnight cultures were diluted 1:100 into fresh media containing erythromycin and grown until an OD₅₉₅ of 0.5 was reached; 10 µM cadmium chloride hemi (pentahydrate) (Sigma, St. Louis, MO) was added to induce intron production for 90 min. The cells were then selected on tryptic soy agar (TSA) plates containing erythromycin. Colony PCR was performed using EBS2 as the forward primer and a SrtA gene sequence downstream of the insertion site, srtA-R (5'-TTTGAAGTTCTGTAGCTACAAAGATT-3'), as the reverse primer. The colonies with a disrupted gene due to intron insertion had PCR products of 1.3 kb. The temperature sensitive plasmid was then eliminated from the cell by incubating the confirmed gene-disrupted mutant strain at 43 °C in antibiotic-free media. As a result the generated strain contained a permanently disrupted SrtA gene.

Southern blot of *srtA*[−] genomic DNA

Southern blot was performed to ensure that the intron insertion was unique to the SrtA gene. Restriction digestion enzymes Spe I, Nsi I, Nde I and Sph I (New England Biolabs, Beverly, MA) were used to fragment genomic DNA. Next, the sample was resolved by electrophoresis on a 0.8% agarose gel, and transferred onto a Hybond-N⁺ nylon membrane (Amersham Bioscience, Piscataway, NJ) by capillary transfer. DIG high prime DNA labeling and detection starter kit (Roche, Indianapolis, IN) was then

used to label the probe DNA and detect the hybridization. DIG labeled intron sequence was PCR amplified using DIG-dUTP. Only one nucleic acids band of 2.5 kb was detected by DIG antibody, which confirms that the intron sequence was only inserted into the SrtA gene.

Construction of SrtA wild-type and mutant protein expression vectors

Primers designated as NcoI-SrtA-F (5'-CATGCCATGGG TCTATGGTTTGTGGTTCATAAG-3') and XhoI-SrtA-R (5'-CCGCTCGAGTTTGAAGTTCTGTAGCTACAAAGATT-3') were used to amplify the *srtA* sequence including its endogenous promoter from genomic DNA of *S. aureus* strain RN4220 by the polymerase chain reaction. The DNA fragments were then digested with Nco I and Xho I (New England Biolabs, Beverly, MA) and cloned into the pET28b expression vector (Novagen, La Jolla, CA) to generate the construct pET28-P-SrtA. Primers designated SphI-SrtA-F (5'-ACATGCATGCGTCTATGGTTTGTGGTTCATAAG-3') and KpnI-P2T-R (5'-CGGGTACCTTACTTGTCTGTCATCGTCTTTGTAGTCGTGGTGGTGGTGGTGGTG-3') were used to copy SrtA sequence along with its N-terminus promoter and C-terminus FLAG tag. The DNA fragment was digested with Hind III and Kpn I (New England Biolabs, Beverly, MA) and cloned into the pNL9164 shuttle vector (Sigma-Aldrich, St. Louis, MO)^{23,24} to generate the construct pT-P-SrtA-His6-FLAG.

The following six primers designated as N132AF (5'-CCGAAGTATCAATTTACAGCTCTTAAAGCAGCCA-AAAAA-3'), N132AR (5'-TTTTTTGGCTGCTTTAAGAGCT GTAAATTGATAGTTCGG-3'), K137AF (5'-CAAATCTT AAAGCAGCCGCAAAAGGTAGTATGGTGTAC-3'), K137 AR (5'-GTACACCATACTACCTTTTTCGGCTGCTTTAA GATTG-3'), Y143AF (5'-GCCAAAAAAGGTAGTATG GTGGCCTTTAAAGTTGGTAATG-3') and Y143AR (5'-CATTACCAACTTTAATGGCCACCATACTACCTTTTTT-GGC-3') were obtained from Invitrogen to generate the mutations N132A, K137A and Y143A respectively. Site-directed mutagenesis was performed as described in the Stratagene protocol²⁵ using pT-P-srtA-His6-FLAG as the template plasmid. Triple mutation mutant SrtA_{NKY} was generated by three-step site-directed mutagenesis.

The SrtA_{ΔN59} expression vector was generated as previously described.¹⁸ SrtA_{ΔN59, NKY} was then generated using the same protocol and primers by using SrtA_{NKY} expression vector as the template.

Expression and purification of SrtA proteins

The pT-P-srtA-His6-FLAG (wild-type or mutant) construct was transformed into *S. aureus* SrtA knockout strain *srtA*[−]. The pT-P-SrtA-His6-FLAG transformed cells were grown in 5 mL of Tryptic Soy Broth media supplemented with 10 µg/mL erythromycin (Sigma, St. Louis, MO) at 32 °C overnight. The cells were harvested and treated with 1 µg/mL lysostaphin (Sigma, St. Louis, MO) at room temperature for 1 h before lysed under native conditions using sonication. SrtA proteins were then purified from cell lysate using the QIAexpression kit according to the manufacturer's protocol (Qiagen, Valencia, CA).²⁶ The concentration of protein was

determined using Bradford reagent (Pierce Biotechnology, Rockford, IL). *In vitro* expression and purification of SrtA_{ΔN59} and SrtA_{ΔN59, NKY} was the same as previously described.¹⁸

Chemical cross-linking in *S. aureus*

S. aureus cells were cultured as described above. EDC (1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride, Sigma, St. Louis, MO) was added to the media to a final concentration of 0.7 mg/mL. Cells were harvested 60 minutes later and analyzed as described above by SDS-PAGE and Western blot.

Polyacrylamide gel electrophoresis and Western blot analysis

Proteins were analyzed on a 14% SDS polyacrylamide gel under denaturing conditions, and visualized by Coomassie Blue staining. For western blot analysis, proteins were first transferred onto a nitrocellulose membrane (Hybond-C Extra, Amersham Bioscience, Piscataway, NJ) in a Tris-glycine buffered electrophoresis tank. The membranes were then probed with an anti-FLAG M2 primary antibody (Sigma, St. Louis, MO) or an anti-sortase A primary antibody (ab13959, Abcam Inc., MA) and a horseradish peroxidase-conjugated goat anti-mouse or goat anti-rabbit secondary antibody (Bio-Rad, Hercules, CA). The Western Blotting Detection Reagent (GE healthcare, UK) was applied to visualize the signals, which were detected by exposing the membrane to BioMax Light Film (Eastman Kodak Company, Rochester, NJ).

Protein in-gel digestion and peptide fingerprint mapping by MS

The proposed dimer and monomer bands of SrtA were sliced from an SDS-polyacrylamide gel and subjected to in-gel trypsin digestion. Proteins were then extracted from the gel and analyzed by MALDI-TOF mass spectrometry and peptide fingerprint mapping analyses in the Analytical Instrumentation Core Facility at the University of Texas at Austin.

Circular dichroism spectrum

All the measurements were repeated three times, and each data point was recorded in triplet. Circular dichroism (CD) studies were conducted on a Jasco J-810 spectropolarimeter (Jasco International Co., Ltd. Tokyo, Japan), equipped with a Jasco PFD-425S temperature control system as described earlier.²¹ Briefly, SrtA_{ΔN59} wild-type and SrtA_{ΔN59, NKY} were dialyzed into activity assay buffer (50 mM Tris-Cl, 150 mM NaCl, 5 mM CaCl₂, pH 7.5) and diluted to a concentration of 39 μM. The recorded spectra were analyzed using the Dichroweb server²⁷ to get an estimation of the composition of the secondary structures. The NRMSD parameter was used to evaluate the fitness of various methods.²⁸ An NRMSD value less than 0.1 indicates that the predicted spectra and the experimental spectra are in close agreement.²⁹

Quantitative analysis of cell surface displayed SPA

Overnight cultures of RN4220, *srtA*⁻, *srtA*⁻+*srtA*, *srtA*⁻+*srtA*_{NKY} were inoculated 1:1000 in fresh TSB and grow till log phase (OD₆₀₀=0.6). 3 mL of each culture was collected by centrifuge at 13 × 10³ r/min for 10 min and washed with Dulbecco's Phosphate Buffered Saline (PBS; Sigma, St. Louis, MO). The cell pellet was then re-suspended in blocking buffer (5 mg/mL BSA in PBS solution) for 10 minutes at room temperature. After blocking, the cell pellet was collected and incubated with labeling buffer, 1 μL IgG-cy3 (C2181, Sigma, St. Louis, MO) in 500 μL blocking buffer for 1 hour at room temperature in the dark. After three washes with blocking buffer, the cell pellet was re-suspended in PBS and analyzed on a FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ) by gating 10 × 10³ *S. aureus* cells per sample based on forward and side scatter. Cell-associated fluorescence was measured in fluorescence channel 2 (FL2-H) to detect Cy-3 fluorescence. Un-labelled RN4220 cells were used as an auto-fluorescence background control and a gate for fluorescence negative population. An estimate of the amount of SPA on cell surface was calculated by multiply the percentage of Cy-3-positive cells with the mean fluorescence intensity of the positive population to obtain the surface protein A (SPA) unit.

HEK293H cell culture

The human embryonic kidney cell line 293H (HEK293H; Invitrogen, Carlsbad, California) was grown in Dulbecco's Modified Eagle's Medium (DMEM; Sigma, St. Louis, MO)/10% Fetal Bovine Serum (FBS; Atlas Biologicals, Fort Collins, CO) at 37°C, 5% CO₂ prior to plating. One day prior to invasion assays, 1.5 × 10⁶ cells were seeded in 6-well plates (Corning Incorporated, Corning, NY) yielding weakly confluent monolayers (3 × 10⁶ cells/well) at the time of invasion.

Labeling of *S. aureus* cells

S. aureus cells (50 mL with OD₆₀₀ 0.6) were washed three times with cold, sterile PBS to reduce aggregation, suspended in 10 mL 0.5 μM 5-(6)-carboxyfluorescein-succinimidylester (CFSE; Molecular Probes, Eugene, OR) in PBS, and incubated for 60 min in 37°C shaker. CFSE-labeled bacteria were washed three times with PBS and protected from direct light exposure.

Invasion assays and flow cytometry

For invasion assays, 3 × 10⁶ HEK293H cells in 6 cm² culture dishes were mixed with the CFSE-labeled bacterial cells at a MOI of 20. The mixture was incubated in DMEM/10% FBS at 37°C for 60 min. Bacterial invasion was terminated by incubation with 300 μg/mL gentamicin (Sigma, St. Louis, MO) in DMEM/10% FBS for 30 min at 37°C to lyse the bacterial cells that fail to enter into the host mammalian cells. The infected mammalian cells were then washed three times with PBS and suspended by treating with trypsin-EDTA solution for 1 min (Sigma, St. Louis, MO). Suspended cells were then incubated with 1 mL fixing

solution (paraformaldehyde solution 4% in PBS; USB Corporation, Cleveland, OH). Samples were kept on ice and dark until analysis. To eliminate signals from extracellular *S. aureus*, trypan blue solution (0.4%; Sigma, Taufkirchen, Germany) was added to a final concentration of 0.2% right before analysis. Samples were analyzed on a FACSCalibur Flow Cytometer by gating 10×10^3 HEK293H cells per sample based on forward and side scatter. Cell-associated fluorescence was measured in fluorescence channel 1 (FL1-H) to detect CFSE fluorescence. An estimate of the amount of internalized bacteria was calculated by multiply the percentage of CFSE-positive cells with the mean fluorescence intensity of these cells to obtain the uptake index (u.i.).

Results

Expression of wild-type SrtA protein in *S. aureus* RN4220 and *srtA* knockout strain

We first analyzed the endogenous expression of full-length SrtA protein in *S. aureus* RN4220 by using denaturing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to separate the cell lysate. A western blot was carried out to probe SrtA with a commercial anti-SrtA polyclonal antibody as described previously.³⁰ Three bands were detected (supplement, Figure S1). The bottom protein band with molecular weight of 28 kDa had the size corresponding to the full-length SrtA monomer. Two additional bands with sizes about 50 kDa and 55 kDa cannot be attributed to SrtA protein, due to the poor selectivity of the antibody. To minimize the interference from non-specific bands, specific epitope tags fused to the C-terminal end of SrtA are necessary to monitor its distribution in *S. aureus* with high fidelity and facilitate its purification as well.

In order to achieve this, we generated a SrtA knockout strain, *srtA*⁻, from *S. aureus* strain RN4220 using TargeTron™ Gene Knockout System (Sigma-Aldrich) which allows for the permanent disruption of the *srtA* gene on bacterial genomic DNA through the insertion of group II introns.²² We confirmed the insertion of the intron into the *srtA* gene by Southern blot, and the absence of SrtA expression in *srtA*⁻ by western blot (Figure S1). The lower band of 28 kDa became absent from *srtA*⁻ cell lysate while it was present in the cell lysate from RN4220 strain.

Next, we used the knockout strain *srtA*⁻ as a host to express a His6-FLAG-tagged full-length SrtA (SrtA-His6-FLAG). Plasmid pT-P-*srtA*-His6-F was created containing the endogenous *srtA* promoter, full-length *srtA* gene followed by a sequence of His6-FLAG epitope tag on its C-terminus. This plasmid was transformed into the *srtA*⁻ cells to afford *srtA*⁻+*srtA* strain. The expression of SrtA-His6-FLAG in *srtA*⁻+*srtA* strain was probed using anti-FLAG antibody and SrtA monomer band with the molecular weight of 28 kDa was observed (Figure 1(a)). Expression SrtA-His6-FLAG in *srtA*⁻+*srtA* strain can also be detected with the anti-SrtA polyclonal antibody (Figure S1). These experiments indicate that the wild-type SrtA was successfully knocked out in the *srtA*⁻ strain and replaced with a SrtA-His6-FLAG protein in *srtA*⁻+*srtA* strain.

The expression level of SrtA-His6-FLAG is close to the level of the endogenous SrtA in RN4220 (Figure S1). In this experiment, we measured the total protein concentration in the cell lysate and loaded the same amount of protein into each lane. The intensities of the SrtA protein bands from RN4220 and *srtA*⁻+*srtA* were used to estimate the relative protein concentrations between the endogenous and the recombinant SrtA proteins, in this case, having the similar integral intensities (Lanes RN4220 and *srtA*⁻+*srtA*, Figure S1).

Figure 1 Chemical cross-linking of SrtA protein in *S. aureus*. Full-length SrtA were fused with FLAG tag on C-terminus and expressed in *S. aureus*. Cell lysate was analyzed by SDS-PAGE and proteins were visualized by western blotting with anti-FLAG M2 antibody (a). Cells were treated with EDC cross-linking reagent or not before harvesting. SrtA protein purified from EDC treated cells were resolved by SDS-PAGE and visualized by Coomassie Blue staining (b). Dimer, monomer and degradation bands as labeled were identified by mass spec data

Chemical cross-linking of SrtA expressed in *S. aureus*

A chemical cross-linking experiment was executed to test the SrtA dimerization on the cell membrane. EDC (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide), a compound that couples carboxylic acid groups with primary amines was used to stabilize the interacting protein oligomers.³¹ The cell culture of *srtA*[−]+*srtA* was briefly treated with EDC prior to harvesting, resolved on SDS-PAGE, and visualized by western blot using anti-FLAG monoclonal antibody. In addition to the 28 kDa monomer band, another strong 56 kDa band was detected, which corresponded to the size of SrtA dimer (Figure 1(a)). In order to characterize the new 56 kDa protein resulting from EDC cross-linking, full-length SrtA-His6-FLAG, expressed and cross-linked in *srtA*[−], was purified from *S. aureus* by C-terminal His6 tag, separated on SDS-PAGE and stained with Coomassie Blue (Figure 1(b)). Two protein bands of interest with molecular weight of 28 kDa and 56 kDa were excised from the gel and subjected to in-gel trypsin digestion followed by MALDI-TOF and peptide fingerprint mapping analyses. Mass spectrometry results identified sortase A as the component of both the 28 and 56 kDa protein bands with Mascot Search scores of 607 and 357, respectively, indicating these species representing monomeric and dimeric forms of the enzyme. In the 56 kDa band, there was another protein identified as putative acetyl-CoA transferase with a molecular weight of 58 kDa. No higher molecular-weight band corresponding to the size of hetero-dimer between this protein and SrtA was observed. It is unlikely that the 56 kDa protein band is the cross-linked product between *srtA* and acetyl-CoA transferase. Due to the common technical challenge of purification of membrane proteins, this acetyl-CoA transferase is most likely a trace amount of contaminant that happens to co-migrate with sortase A dimer in SDS-PAGE. Thus, current data suggest, with high confidence, that the 56 kDa band is the homodimer band of SrtA. Compared to Figure 1(a), a band was detected with molecular weight of about 22 kDa, with the reduced amount of SrtA monomer (28 kDa) and SrtA dimer (56 kDa) (Figure 1(b)). Mass spectroscopic analysis confirmed with a Mascot Search score of 557 that this additional protein band of 22 kDa is a degradation product of full-length SrtA.

Site-directed mutagenesis to disrupt SrtA dimerization on the cell membrane

Site-directed mutagenesis was applied to create a full-length SrtA mutant incapable of forming a dimer on cell membrane. Previously, three SrtA mutants in truncated forms have been shown to exist as a monomer *in vitro*.²¹ Each of these mutant proteins contained a single point mutation of one amino acid residue on the surface (N132A, K137A and Y143A, respectively). The mutation was also introduced on the same site in full-length SrtA-His6-FLAG to afford three SrtA mutants, N132A, K137A and Y143A. Each of these three mutants was expressed in *S. aureus* and the SrtA knockout strain *srtA*[−] respectively. Their cultures were treated with EDC before harvesting. Cell lysates were collected and separated on SDS-PAGE followed by Western blot (Figure 2). The 28 kDa SrtA

Figure 2 Western blot analysis of chemical cross-linking of full-length SrtA wild-type and mutant proteins on cell membrane. *S. aureus* cells over-expressing SrtA wild-type (lane 1) and mutant proteins (lane 2 for N132A, 3 for K137A, 4 for Y143A, 5 for SrtA_{NKY}) were treated with EDC before harvesting. All the cell lysate samples were analyzed simultaneously on a single SDS-PAGE followed by Western blot probed with anti-FLAG antibody. Total protein concentration of cell lysate was measured and equal amount of protein was loaded for each lane. The final figure is the result of remove the "empty" lanes between samples that prevent the over-flow. The "smile" pattern of the lane of "triple mutation" is common to the protein separation at the end of SDS-PAGE gel

monomer band was observed for all three mutant proteins and the intensity of the bands was comparable to the monomer band of the wild-type protein, confirming the same expression level between those mutant and wild-type SrtA proteins. However, the cross-linked dimer bands of 56 kDa were still observed in the cell lysates of all three SrtA mutant-expressing strains. Thus, the same single-mutations that disrupted the dimerization of SrtA catalytic domain were unable to break the dimerization of full-length SrtA expressed on *S. aureus* cell membrane. A full-length SrtA-His6-FLAG mutant to harbor all three mutations (N132A, K137A and Y143A) simultaneously (SrtA_{NKY}) was created and expressed in *srtA*[−]. This strain was also analyzed by EDC-mediated cross-linking experiment. As shown in Figure 3, the 28 kDa SrtA monomer band of SrtA_{NKY} was observed with similar intensity compared to the wild-type protein and other mutant proteins, indicating that the triple mutant SrtA has the similar expression level as the other SrtA proteins in *S. aureus*. However, here the 56 kDa dimer band was absent from this strain, clearly indicating the inability of SrtA_{NKY} to form a dimer on the cell membrane. Thus, disruption of dimerization was achieved by replacing all three residues (N132, K137, and Y143) with alanine residues.

To assess the structural changes upon mutations, circular dichroism (CD) analyses were performed with wild-type SrtA_{ΔN59} and SrtA_{ΔN59,NKY} mutant, both are the extra-cellular catalytic domains and much easier to purify.^{13,32} The recorded spectra in millidegrees of ellipticity (θ) were converted to mean residue ellipticity [θ] as indicative of changes of secondary structure in proteins. The estimated contents of secondary structure of SrtA_{ΔN59,NKY} are derived from the Dichroweb server.²⁷ A NRMSD fit parameter of 0.051 was obtained for SrtA_{ΔN59,NKY}, indicating the consistency of the calculated secondary structures and the experimental measurement. SrtA_{ΔN59,NKY} contains 8% α-helix, 37% β-sheet, 23% β-turn, and 31% random loop, which is

Figure 3 Quantification of SPA display on *S. aureus* cell surface by flow cytometry. *S. aureus* cells were incubated with CY-3-IgG for 60 min at room temperature, suspended in PBS and analyzed by flow cytometry. The SPA unit was calculated. The bars represent the mean values of the SPA unit for RN4220, *srtA*⁻, *srtA*⁻ + *srtA*, and *srtA*⁻ + *srtA*_{NKY} as observed in three independent experiments. The average SPA units and the standard deviation were given on top of each bar, with p-value < 0.01 for *srtA*⁻, *srtA*⁻ + *srtA* and 0.81 for *srtA*⁻ + *srtA*_{NKY}.

comparable to wild-type SrtA_{ΔN59}. These data suggests the global secondary structure of SrtA_{ΔN59,NKY} remains largely unchanged after mutations, but the local structure at the dimerization interface may alter in such a way to disrupt the dimerization.

***S. aureus* with monomeric SrtA mutant displays more adhesive proteins on its cell surface than wild-type SrtA**

We first tested whether the functional display of staphylococcal cell surface adhesins, such as surface protein A (SPA), may be affected by the oligomerization states of SrtA on the cell surface. SrtA is responsible for displaying various LPXTG motif-bearing proteins that were identified as bacterial adhesion proteins (Spa, FnBP-A, FnBP-B, ClfA, ClfB, Cna, SdrC, SdrD, SdrE) onto the surface of *S. aureus*.¹⁰ Since the activity of SrtA directly controls the efficiency of anchoring these adhesion proteins, assembly of functional SPA proteins on cell surface can be quantified to measure SrtA's activity on the cell membrane. *Staphylococcal* cells were incubated with SPA protein's specific ligand, CY3-IgG, which is coupled with a CY-3 fluorescent dye. The binding of IgG with SPA on the bacterial surface was then quantified by the detection of cell-associated fluorescence in a flow cytometer. In this method, not only the percentage of fluorescence labeled cells can be determined, the strength of the fluorescent signal also provides the estimation of the relative average amount of SPA proteins displayed on each cell. The SPA unit was then introduced to provide the correlated total amount of SPA proteins on cell surface of different strains and used to compare the activities of SrtA on their cell surface. It is calculated by multiplying the percentage of fluorescent cells by the mean fluorescence intensity of the fluorescent population. To validate this concept, *S. aureus* strain RN4220 and SrtA knockout strain *srtA*⁻ were both labeled with CY-3-IgG. As a result, CY3-labeled RN4220 was found in a large proportion, about 46.6% of total cells

sorted, whereas only 8.3% of *srtA*⁻ cells were fluorescent. Averaged from three independent repeats, the calculated SPA unit of *srtA*⁻ was 3.0, while the SPA unit of RN4220 was 16.1. The difference of the total amount SPA protein on cell surface between RN4220 and *srtA*⁻ resulted from the availability of active SrtA on the bacterial surface and consistent with the previous observation that SrtA plays a critical role in displaying SPA on the *S. aureus* cell surface.³³

Using this assay, the SPA units of *S. aureus* strains harboring the vector-expressed wild-type SrtA and monomeric SrtA mutant protein were measured (Figure 3). After the 1-h incubation with IgG-CY3, about 29.4% of *srtA*⁻ + *srtA* cells were fluorescent with a calculated SPA unit of around 8.6. In contrast, about 46.2% of *srtA*⁻ + *srtA*_{NKY} cells were fluorescent with SPA unit of 15.2 (Figure 3). Both *srtA*⁻ and *srtA*⁻ + *srtA* showed a statistically significant difference ($p < 0.01$) in SPA display when compared to wild type RN4220 strain. Over-expression of sortase A in *srtA*⁻ + *srtA* affords the shifting the equilibrium towards dimerization on the cell membrane. However, the SPA level from *srtA*⁻ + *srtA*_{NKY} cells is not statistically different from RN4220 strain (p value = 0.81), suggesting that a monomeric form of sortase A can restore the display of SPA on the cell surface at a level comparable to the wild type *S. aureus* RN4220. This result demonstrated that *srtA*⁻ strain harboring monomeric SrtA on its membrane (*srtA*⁻ + *srtA*_{NKY}) achieves more efficient display of cell surface protein SPA than the strain harboring expressed wild-type SrtA (*srtA*⁻ + *srtA*). In a size-exclusion chromatography experiment, the ratio between dimeric and monomeric SrtA in *srtA*⁻ + *srtA* cells has been quantified as 2.94: 1 (Supplemental Materials, additional methods and results). The significant difference in the cell surface display efficiency suggested the functional difference between dimeric SrtA and monomeric SrtA mutant on the cell membrane.

S. aureus with monomeric SrtA is more invasive than *S. aureus* with wild-type SrtA

A quantitative invasion assay was set up to detect two other cell surface proteins displayed by SrtA and evaluate the biological effects of SrtA dimerization on the cell membrane^{34–36} (Figure 4(a)). Among the various LPXTG proteins, two fibronectin-binding proteins (FnBP-A and FnBP-B) have been demonstrated to be sufficient for *S. aureus* to enter non-professional phagocytes *in vitro*, such as various epithelial and endothelial cells.^{37–39} They bind and recruit the extra-cellular matrix protein fibronectin (Fn) from the host environment or media onto the bacterial surface. Cellular surface bound Fn is then recognized by the principal host cell fibronectin receptor, the integrin $\alpha_5\beta_1$. The fibronectin-bridge via FnBP and integrin $\alpha_5\beta_1$ is the major route of *S. aureus* internalization. In *S. aureus* and various Gram-positive strains, the inactivated SrtA protein (e.g. *srtA*[−]) would abolish the proper display of adhesion proteins and decrease the efficiency of the strain in invading mammalian cells.^{4–6,30,40,41} Activity of SrtA determines the efficiency of anchoring these adhesion proteins and the total number of *S. aureus* cells that invade the host mammalian cells. A cell-based invasion assay was set up based on this

mechanism.⁴² The mammalian cells were infected with fluorescein-labeled bacteria and the suspension of infected host cells was applied to flow cytometry to measure fluorescence from internalized *S. aureus*. Two parameters are necessary to be taken into consideration to quantify the invasion efficiency, the percentage of infected cells can be determined and the strength of the fluorescent signal that provides the estimation of the relative amount of internalized bacteria in each sample.⁴² The uptake index (u.i.) was introduced and calculated by multiplying the percentage of fluorescent cells by the mean fluorescence intensity of the fluorescent population.⁴² The u.i. was then used to indicate the biological activity of SrtA on the cell membrane. *S. aureus* strain RN4220 which contains endogenous SrtA and *SrtA* knockout strain *srtA*[−] were both labeled with carboxyfluorescein succinimidyl ester (CFSE) and used to infect the host HEK293H cells. Internalized RN4220 was found in a large proportion, about 40% of HEK293H cells, whereas intra-cellular *srtA*[−] strain was only detected in about 8% of HEK293H cells (Figure 4(b)). Averaged from five independent repeats, the calculated uptake index (u.i.) of *srtA*[−] is 1.5, which is about 10% of the u.i. of 14.8 calculated for RN4220. The difference of infections between

Figure 4 Quantification of *S. aureus* invasion in HEK293H by flow cytometry. (a) Cartoon representation of invasion assays. (b) HEK293H cells were infected for 60 min with CFSE-labeled *S. aureus* RN4220 and *srtA*[−] respectively, suspended in fixing solution with 0.2% trypan blue and analyzed by flow cytometry. The original dot plots of a representative experiment were shown. (c) HEK293H cells were infected and analyzed as in (b). The uptake index (u.i.) was calculated. The bars represent the mean values of the u.i. for RN4220 and *srtA*[−] as observed in five independent experiments. The average u.i. number and the standard deviation were given on top of each bar. (A color version of this figure is available in the online journal)

RN4220 and *srtA*⁻ resulted from the availability of functional FnBPs on cell surface and the abolishment of SrtA function in *srtA*⁻. This data is consistent with the previous discovery that SrtA plays a critical role in *S. aureus* infection and is a potential drug target.³³ It also indicates that the uptake index (u.i.) is a reliable measurement of the bacterial internalization and an applicable indicator for SrtA's activities for anchoring virulence factors on the cell membrane.

Using this assay, the uptake indexes (u.i.) of *S. aureus* strains harboring wild-type SrtA and monomeric SrtA mutant protein were measured (Figure 5). To keep the genetic consistence among the strains, SrtA knock out strain *srtA*⁻ was used as the host strain throughout the assay and served as the baseline for the uptake index measurement. After 1-h infection with the host mammalian cells, *srtA*⁻+*srtA* cells were detected in about 20% of the infected mammalian cells (Figure 5(a)), with a calculated u.i. of around 5.2 (Figure 5(b)). *srtA*⁻+*srtA*_{NKY} cells were detected in about 45% of the infected cells with u.i. of 14.6 (Figure 5(a) and (b)), which has majority of molecules in dimeric form (supplement Figure S2 and additional

methods and materials). These results demonstrated that *srtA*⁻ strain harboring monomeric SrtA on its membrane has higher invasion efficiency than the strain harboring wild-type SrtA, which has majority of molecules in dimeric form.

Discussion

Dimerization of truncated SrtA_{Δ59} has been reported and studied *in vitro*.^{18–21} In this paper, we report the observation of the dimerization of the full length SrtA expressed in *S. aureus*. As a supporting evidence and critical reagent for the study, a SrtA mutant has also been created to exist only as monomer on the cell membrane. It is interesting to notice the difference of binding affinity of SrtA between in solution and on cell membrane. For *in vitro* expressed catalytic domain SrtA_{Δ59}, mutation of any single residue of the following three amino acids N132, K137 and Y143 to alanine is able to disrupt dimerization. However, when SrtA is expressed in its full-length form on *S. aureus* membrane, the same single mutation of these residues fails to

Figure 5 *S. aureus* cells with monomeric SrtA is more invasive than the cells with wild-type SrtA. (a) HEK293H cells were infected for 60 min with CFSE-labeled *S. aureus* *srtA*⁻, *srtA*⁻ + *srtA*, and *srtA*⁻ + *srtA*_{NKY} respectively, suspended in fixing solution with 0.2% trypan blue and analyzed by flow cytometry. The original dot plots of a representative experiment were shown. (b) HEK293H cells were infected and analyzed as in (a). The uptake index (u.i.) was calculated. The bars represent the mean values of the u.i. for *srtA*⁻, *srtA*⁻ + *srtA*, and *srtA*⁻ + *srtA*_{NKY} respectively as observed in five independent experiments. The average u.i. number and the standard deviation were given on top of each bar with p-value < 0.01 for *srtA*⁻, *srtA*⁻ + *srtA* and 0.88 for *srtA*⁻ + *srtA*_{NKY}. (c) Cell lysate of *srtA*⁻, *srtA*⁻ + *srtA*, and *srtA*⁻ + *srtA*_{NKY} were analyzed by SDS-PAGE and a western blot to quantify the expression of SrtA on cell membrane. EDC was added to stabilize the dimerization between SrtA molecules

completely disrupt SrtA homo-dimer. Only SrtA_{NKY} mutant accumulates the effects of all three mutations and significantly reduces its dimerization. Thus, the full length SrtA forms stronger dimer on the cell membrane than its truncated form SrtA_{Δ59} in solution with the dissociation constant measured at 55 μM.¹⁸ Several factors may contribute to the enhanced dimerization of full-length SrtA expressed on *S. aureus* cell membrane. The transmembrane domain of SrtA would provide additional hydrophobic interaction. It might interact with other molecules within the membrane so that the intermolecular association between SrtA molecules would be reinforced by the protein complex. This type of association for a multi-enzyme complex has been proposed and experimentally supported in the case of bacterial peptidoglycan biosynthesis holoenzyme.⁴³ It is also likely that the full-length SrtA expressed in *S. aureus* behaves differently from that expressed in *E. coli*, for example, we observed that SrtA expressed in *S. aureus* is more unstable than that in *E. coli* (supplemental Figure S2).

The similar phenomena that compared to its truncated form, full-length protein expressed on cell membrane forms stronger dimer to activate its activity has been reported other bacterial cell membrane proteins, such as Outer Membrane PhosphoLipase A (OMPLA) in Gram-negative bacteria,⁴⁴ which implies the biological importance for the dimerization of SrtA *in vivo*.

In order to study the roles of sortase A dimerization on *S. aureus* cell membrane, we assessed the biological activities of monomeric and dimeric SrtA on *S. aureus* cell membrane by measuring the efficiency of cell surface display of SrtA substrates, SPA and FnBPs, via an established *in vitro* IgG fluorescent labeling assay and a cell-based invasion assay respectively. It is important to notice that the results from these two different assays are consistent and mutually supportive. In *srtA*[−]+*srtA*_{NKY} strain, SrtA exists as a monomeric enzyme. This strain has more SrtA substrates anchored onto the cell wall (SPA unit of 15.2) and results in more efficient internalization into mammalian cells (u.i. of 14.6), which indicates higher SrtA activity on the cell membrane. On the other hand, majority of SrtA molecules exists as dimer in *srtA*[−]+*srtA* strain (supplemental Figure S2 and additional methods and materials) and under the circumstance the enzyme becomes less active than monomeric SrtA mutant on *S. aureus* cell membrane, demonstrated by a SPA unit of 8.6 and a u.i. of 5.2. In both strains of *srtA*[−]+*srtA* and *srtA*[−]+*srtA*_{NKY}, the expression of the *srtA* genes is regulated by the same endogenous *srtA* promoter. The protein concentrations on the cellular membrane are at the same level when detected with Western blot. SrtA_{Δ59,NKY} has lower substrate cleavage activity compared to wild-type SrtA_{Δ59} *in vitro* when concentrations of both enzymes in solution are much lower than the dissociation constant *K*_d, 55 μM (supplemental materials). Under these conditions, both SrtA_{Δ59,NKY} and SrtA_{Δ59} exist as monomers. The exact reason for the reduction of activity of SrtA_{Δ59,NKY} compared to SrtA_{Δ59} is not clear at this stage, but it could be the conformational change caused by the mutations. Nevertheless, this data excludes the possibility that the higher invasion efficiency of

srtA[−]+*srtA*_{NKY} is caused by the higher enzymatic activity of SrtA_{NKY} itself. Hence, the difference of invasion activities between these two strains is highly likely related to the difference in oligomerization state of SrtA on cell membrane. In other words, these data suggested that dimerization of SrtA between wild-type proteins greatly suppresses its enzymatic activity on cell membrane.

Conclusion

We have provided the evidence that SrtA exists in a monomer-dimer equilibrium in *S. aureus*. It has been shown in several different biological systems that protein dimerization is used for the efficient and accurate control of many distinct functions.⁴⁵ It confers various structural and functional advantages to proteins, such as improved stability, control over the specificity of active sites, and increased complexity. For sortase A, Suree N., *et al.* reported using NMR that the β6/β7 loop undergoes conformational changes from a non-productive and disordered state in apo-SrtA to a “closed”, immobilized and productive state in substrate-bound enzyme.⁴⁷ Interestingly, their result is in agreement with our molecular dynamics simulation data of SrtA_{Δ59} and SrtA_{Δ59,NKY} enzymes (data not shown). The triple mutations on SrtA_{Δ59} induced a “fixed” β6/β7 loop structure (less flexible) in SrtA_{Δ59,NKY} monomer that is very similar to the substrate-bound induced “closed” β6/β7 loop structure from Suree’s publication. Thus, we propose that sortase A’s enzymatic activity is regulated by monomer-dimer equilibrium on the bacterial membrane, possibly by affecting the configurations of SrtA active site. The wild type SrtA when exists as dimer on the cell membrane has a stretched and disordered β6/β7 loop. Upon the binding of substrate LPXTG sequence, SrtA dimer has to dissociate and the resulting monomeric SrtA can reorganizes its β6/β7 loop to a “closed” and active conformation for substrate binding and catalysis, therefore affords higher *in vivo* activity. Our results suggest that SrtA monomer in the “closed” conformation is the accurate drug target to inhibit for drug discovery.⁴⁶

The monomer-dimer equilibrium and biogenesis of sortase A are possibly regulated by other components involved in SrtA pathway, given its critical role in microbial pathogenesis. Identification of these regulatory factors will provide significant academic and therapeutic interests by itself. Overall, the observations that SrtA forms dimer on cell membrane and its monomeric mutant was associated with better biological function as described here sheds light on the mechanisms of sortase A function *in vivo* and may inspire the development of anti-infective agents to target sortase A dimer-monomer equilibrium.

Author contributions: JZ executed majority of the experiments, obtained data, analyzed and discussed the data with ZZ, and drafted the manuscript. LX executed and trouble-shot some experiments and contributed to the scientific discussions. FJ repeated all the experimental data, and further proved the mechanism by identifying mechanism-based enzyme inhibitors that antagonize the infection. ZZ came up with the hypothesis, designed the set of experiments to

test the hypothesis, analyzed and discussed the experimental data and drew the scientific conclusion. ZZ finalized the manuscript and submitted for publication. ZZ also supervised the JZ, LX and FJ for their daily research operations.

ACKNOWLEDGEMENTS

We would like to thank Dr. Alan Lambowitz and Dr. Jun Yao for kind gift of *S. aureus* strain RN4220 and their help with culturing and manipulation of *S. aureus* cells. We also appreciate Dr. Stony H. Lo and Dr. Marian Person for their mass spectroscopic analyses. We also appreciate Dr. Alexander Horswill for helpful discussions and Katy Moncivais for proofreading the manuscript. The work was supported by The Welch Research Foundation Grant (F1618).

REFERENCES

- Lowy FD. *Staphylococcus aureus* infections. *N Engl J Med* 1998;**339**:520–32
- Applebaum PC. MRSA – the tip of the iceberg. *Clin Microbiol Infect* 2006;**12**:3–10
- Alksne LE, Projan SJ. Bacterial virulence as a target for antimicrobial chemotherapy. *Curr Opin Biotechnol* 2000;**11**:625–36
- Bierne H, Mazmanian SK, Trost M, Pucciarelli MG, Liu G, Dehoux P, Jansch L, Garcia-del Portillo F, Schneewind O, Cossart P. Inactivation of the *srta* gene in *Listeria monocytogenes* inhibits anchoring of surface proteins and affects virulence. *Mol Microbiol* 2002;**43**:869–81
- Bolken TC, Franke CA, Jones KF, Zeller GO, Jones CH, Dutton EK, Hruby DE. Inactivation of the *srta* gene in *Streptococcus gordonii* inhibits cell wall anchoring of surface proteins and decreases in vitro and in vivo adhesion. *Infect Immun* 2001;**69**:75–80
- Mazmanian SK, Ton-That H, Schneewind O. Sortase-catalysed anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. *Mol Microbiol* 2001;**40**:1049–57
- Maresso AW, Schneewind O. Sortase as a target of anti-infective therapy. *Pharmacol Rev* 2008;**60**:128–41
- Ton-That H, Liu G, Mazmanian SK, Faull KF, Schneewind O. Purification and characterization of sortase, the transpeptidase that cleaves surface proteins of *Staphylococcus aureus* at the LPXTG motif. *Proc Natl Acad Sci USA* 1999;**96**:12424–29
- Foster TJ, Hook M. Surface protein adhesins of *Staphylococcus aureus*. *Trends Microbiol* 1998;**6**:484–8
- 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**:760–63
- Navarre WW, Schneewind O. Surface proteins of Gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiol Mol Biol Rev* 1999;**63**:174–229
- Perry AM, Ton-That H, Mazmanian SK, Schneewind O. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. III. Lipid II is an in vivo peptidoglycan substrate for sortase-catalyzed surface protein anchoring. *J Biol Chem* 2002;**277**:16241–48
- Ilangovan U, Ton-That H, Iwahara J, Schneewind O, Clubb RT. Structure of sortase, the transpeptidase that anchors proteins to the cell wall of *Staphylococcus aureus*. *Proc Natl Acad Sci USA* 2001;**98**:6056–61
- Zong Y, Bice TW, Ton-That H, Schneewind O, Narayana SV. Crystal structures of *Staphylococcus aureus* sortase A and its substrate complex. *J Biol Chem* 2004;**279**:31383–89
- Mazmanian SK, Ton-That H, Su K, Schneewind O. An iron-regulated sortase anchors a class of surface protein during *Staphylococcus aureus* pathogenesis. *Proc Natl Acad Sci USA* 2002;**99**:2293–8
- Mandlik A, Das A, Ton-That H. The molecular switch that activates the cell wall anchoring step of pilus assembly in Gram-positive bacteria. *Proc Natl Acad Sci USA* 2008;**105**:14147–152
- Raz A, Fischetti VA. Sortase A localizes to distinct foci on the *Streptococcus pyogenes* membrane. *Proc Natl Acad Sci USA* 2008;**105**:18549–54
- Lu C, Zhu J, Wang Y, Umeda A, Cowmeadow RB, Lai E, Moreno GN, Person MD, Zhang Z. *Staphylococcus aureus* sortase A exists as a dimeric protein in vitro. *Biochemistry* 2007;**46**:9346–54
- Thibodeaux GN, Cowmeadow R, Umeda A, Zhang Z. A tetracycline repressor-based mammalian two-hybrid system to detect protein-protein interactions in vivo. *Anal Biochem* 2009;**386**:129–31
- Umeda A, Thibodeaux GN, Zhu J, Lee Y, Zhang Z. Site-specific protein cross-linking with genetically incorporated 3,4-dihydroxy-L-phenylalanine. *ChemBiochem* 2009;**10**:1302–4
- Zhu J, Lu C, Standland M, Lai E, Moreno GN, Umeda A, Jia X, Zhang Z. Single mutation on the surface of *Staphylococcus aureus* sortase A can disrupt its dimerization. *Biochemistry* 2008;**47**:1667–74
- Yao J, Zhong J, Fang Y, Geisinger E, Novick RP, Lambowitz AM. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of L1.LtrB group II intron splicing. *RNA* 2006;**12**:1271–81
- Charpentier E, Anton AI, Barry P, Alfonso B, Fang Y, Novick RP. Novel cassette-based shuttle vector system for Gram-positive bacteria. *Appl Environ Microbiol* 2004;**70**:6076–85
- Grkovic S, Brown MH, Hardie KM, Firth N, Skurray RA. Stable low-copy-number *Staphylococcus aureus* shuttle vectors. *Microbiology* 2003;**149**:785–94
- Agilent Technologies. 5301 Stevens Creek Blvd, Santa Clara, CA 95051. Instruction Manual Revision D.01. QuikChange Site-Directed Mutagenesis Kit. <http://www.chem.agilent.com/library/usermanuals/Public/200518.pdf>
- Qiagen Inc. *The QIAexpressionist: a handbook for high-level expression and purification of 6XHis-tagged proteins*. June, 2003. <http://www.qiagen.com/us/resources/resourcedetail?id=79caf7d-42fe-4d62-8676-4cfa948c9435&lang=en>
- Lobley A, Whitmore L, Wallace BA. DICHROWEB: an interactive website for the analysis of protein secondary structure from circular dichroism spectra. *Bioinformatics* 2002;**18**:211–12
- Mao D, Wachter E, Wallace BA. Folding of the mitochondrial proton adenosinetriphosphatase proteolipid channel in phospholipid vesicles. *Biochemistry* 1982;**21**:4960–8
- Brahms S, Brahms J. Determination of protein secondary structure in solution by vacuum ultraviolet circular dichroism. *J Mol Biol* 1980;**138**:149–78
- Mazmanian SK, Liu G, Jensen ER, Lenoy E, Schneewind O. *Staphylococcus aureus* sortase mutants defective in the display of surface proteins and in the pathogenesis of animal infections. *Proc Natl Acad Sci USA* 2000;**97**:5510–15
- Staros JV, Wright RW, Swingle DM. Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. *Anal Biochem* 1986;**156**:220–22
- Ton-That H, Schneewind O. Anchor structure of staphylococcal surface proteins. IV. Inhibitors of the cell wall sorting reaction. *J Biol Chem* 1999;**274**:24316–20
- Cossart P, Jonquieres R. Sortase, a universal target for therapeutic agents against Gram-positive bacteria? *Proc Natl Acad Sci USA* 2000;**97**:5013–15
- Ahmed S, Meghji S, Williams, Henderson B, Brock JH, Nair SP. *Staphylococcus aureus* fibronectin binding proteins are essential for internalization by osteoblasts but do not account for differences in intracellular levels of bacteria. *Infect Immun* 2001;**69**:2872–77
- Alexander EH, Hudson MC. Factors influencing the internalization of *Staphylococcus aureus* and impacts on the course of infections in humans. *Appl Microbiol Biotechnol* 2001;**56**:361–66
- Jevon M, Guo C, Ma B, Mordan N, Nair SP, Harris M, Henderson B, Bentley G, Meghji S. Mechanisms of internalization of *Staphylococcus aureus* by cultured human osteoblasts. *Infect Immun* 1999;**67**:2677–81
- Dziewanowska K, Patti JM, Deobald CF, Bayles KW, Trumble WR, Bohach GA. Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells. *Infect Immun* 1999;**67**:4673–78
- Sinha B, Francois P, Que YA, Hussain M, Heilmann C, Moreillon P, Lew D, Krause KH, Peters G, Herrmann M. Heterologously expressed

- Staphylococcus aureus* fibronectin-binding proteins are sufficient for invasion of host cells. *Infect Immun* 2000;**68**:6871–8
39. Sinha B, Francois PP, Nusse O, Foti M, Hartford OM, Vaudaux P, Foster TJ, Lew DP, Herrmann M, Krause KH. Fibronectin-binding protein acts as *Staphylococcus aureus* invasins via fibronectin bridging to integrin $\alpha 5 \beta 1$. *Cell Microbiol* 1999;**1**:101–17
40. Garandeau C, Reglier-Poupet H, Dubail I, Beretti JL, Berche P, Charbit A. The sortase SrtA of *Listeria monocytogenes* is involved in processing of internalin and in virulence. *Infect Immun* 2002;**70**:1382–90
41. Ton-That H, Mazmanian SK, Faull KF, Schneewind O. Anchoring of surface proteins to the cell wall of *Staphylococcus aureus*. Sortase catalyzed in vitro transpeptidation reaction using LPXTG peptide and NH(2)-Gly(3) substrates. *J Biol Chem* 2000;**275**:9876–81
42. Pils S, Schmitter T, Neske F, Hauck CR. Quantification of bacterial invasion into adherent cells by flow cytometry. *J Microbiol Methods* 2006;**65**:301–10
43. Holtje JV. A hypothetical holoenzyme involved in the replication of the murein sacculus of *Escherichia coli*. *Microbiology* 1996;**142**(Pt 8): 1911–18
44. Dekker N, Tommassen J, Lustig A, Rosenbusch JP, Verheij HM. Dimerization regulates the enzymatic activity of *Escherichia coli* outer membrane phospholipase A. *J Biol Chem* 1997;**272**:3179–84
45. Phizicky EM, Fields S. Protein-protein interactions: methods for detection and analysis. *Microbiol Rev* 1995;**59**:94–123
46. Suree N, Yi SW, Thieu W, Marohn M, Damoiseaux R, Chan A, Jung ME, Clubb RT. Discovery and structure-activity relationship analysis of *Staphylococcus aureus* sortase A inhibitors. *Bioorg Med Chem* 2009;**17**:7174–85

(Received December 17, 2014, Accepted March 20, 2015)