May 2019

Engineering Sortase; Activity and Selectivity of New Hybrid and Ancestral Variants of Sortase A

Sarah Struyvenberg
Western Washinton University

Purification and Characterization of Sortase A: an Inquiry into the Evolution and Mechanisms of Action

Western Washington University, Department of Chemistry
Sarah Struyvenberg, Jordan Valgardson, Nick Horvath, John Antos, Jeanine Amacher

Abstract
The emergence of sortase enzyme utilization, specifically with sortase A, has been a key component in novel mechanisms of protein engineering. The central framework behind sortase mediated, site-specific ligation involves recognition of the substrate containing the LPATXG motif by the Sortase A (SrtA) and successive cleavage between the threonine and glycine residues. This in turn generates an acetyl-enzyme intermediate, consequently leading to a reaction with the N-terminal glycine, recreating the native amide bond. This methodology allows researchers to create innovative protein modifications that would otherwise be difficult or impossible under native conditions.[1][2]
The goal of our research is to first, express and purify wild type SrtA from both *S. aureus* ($SrtA_{Staph}$) and *S. pneumoniae* ($SrtA_{Strep}$) to establish a baseline of activity and selectivity (Fig. 10, Fig. 11). Secondly, we will express and purify loop swapped *Streptococcus-aureus* ($SrtA_{Strep\_Swap\_aureus}$) and *Staphylococcus-pneumoniae* ($SrtA_{Staph\_Swap\_Pneum}$) and perform binding assays (K_D and competition experiments) to determine selectivity for these proteins. Thirdly, based on the previous work done on the ancestral sortase reconstruction (Fig. 1, Fig. 2) we will express and purify both the ancestral SrtA for *S. aureus* (ancSrcA_Staph) and *S. pneumoniae* (ancSrtA-Strep) to test the sequence selectivity of these ancestral proteins.
Our current goals include not only binding affinity analysis, selectivity tests, but also the crystallization of the purified loop swapped and ancestral SrtA proteins.

Background
Our research proposal is based on the work of the Antos Lab at WWU. Their primary investigation has encompassed the purification and characterization of activity of sortase homologues. In conjunction with the Antos lab, we are expressing and purifying four new sortase homologues; the two loop swapped SrtA proteins and the ancestral SrtA proteins. Our loop swapped complexes involve switching the two loops highlighted below. Due to the differing selectivity of these two proteins we are interested to explore how a loop swap will effect the overall selectivity. We also would like to investigate how we could possibly find or engineer a more promiscuous sortase enzyme.
Currently we have been able to express and purify both loop swapped proteins (Fig. 4-9) The protocol utilized was taken from previous research done in the Antos lab (Fig. 3). The protocol and results are discussed in the right-hand column.

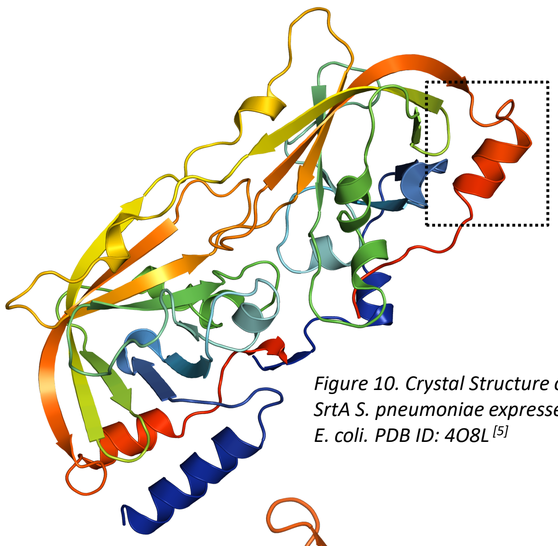

Figure 10. Crystal Structure of SrtA S. pneumoniae expressed in E. coli. PDB ID: 4O8L [5]

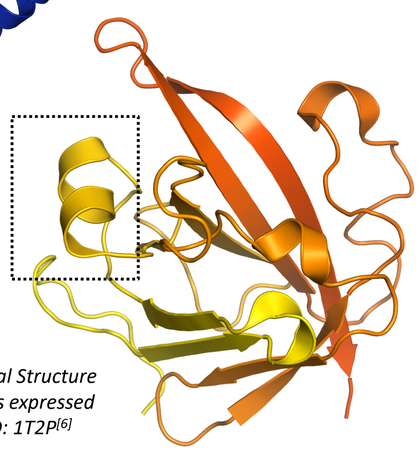

Figure 11. Crystal Structure of SrtA S. aureus expressed in E. coli. PDB ID: 1T2P[6]

Ancestral Sequence Reconstruction
A sequence alignment of sortase sequences was performed by taking NCBI non redundant sortase sequences and using CD-HIT to filter out highly similar (greater then 95% identical sequences). All vs all BLAST was preformed on the remaining sortase sequences, the resulting sortase network then informed the assignment of the sortase groups (aka A,B,C,D,E, and F) by utilizing labeled sortase sequences to assign a class to each grouping. The proteins that clustered as sortase A were then selected. Again highly similar proteins (greater than 90 percent similar) were removed by CD-HIT. The remaining proteins were then aligned via MUSCLE. The multi sequence alignment was then manually curated. Sortase A structures from PDB were structurally aligned and sequence similarity between structure sequences and other sequences in the MSA informed the true alignment of the MSA. A phylogenetic tree was produced from the alignment via phyml and the ancestral sequences were generated via maxml. Nodes preceding the streptococcus pneum. and and staph aureus branches with high support were selected for further analysis.

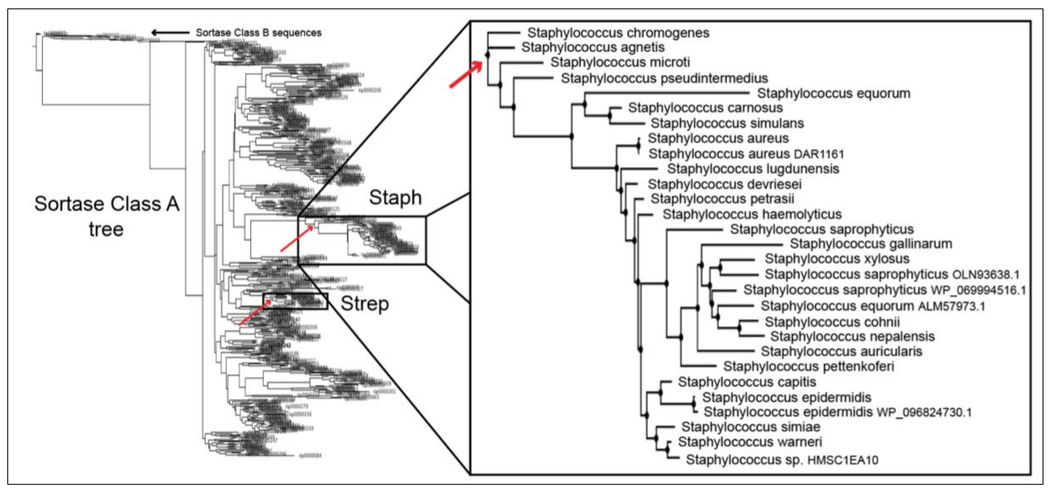

Figure 1. Phylogenetic tree of sequences used for ancestral sequence reconstruction (ASR) of Class A sortase enzymes from Staphylococcus (see red arrow, and zoomed in tree to right) and Streptococcus pneumoniae (see lower red arrow on tree to left). The arrows indicate the nodes that were reconstructed.

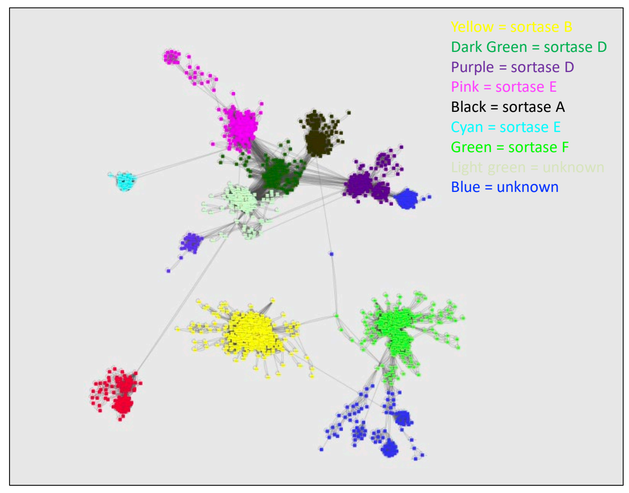

Figure 2. Network analysis of Sortase. Sortase groups organized by class. Resolved via CD-HIT and BLAST, sequences obtained from NCBI.

Methods
Expression and Purification Protocol;

Cells expressed in *E. coli* BL21-DE3 cells → Resuspended cells centrifuged and filtered → Lysate loaded onto His-Bind resin column. Washed with denaturing wash buffer, then eluted with denaturing elution buffer → Eluted fractions collected and rapidly diluted in dilution buffer (100x) → Diluted fractions washed with native wash buffer, eluted with native elution buffer → Size exclusion chromatography (SEC) was preformed, eluted fractions loaded onto SEC column and eluted with running buffer

Binding Assay Overview;
Binding studies will be preformed using fluorescence polarization to test the affinities of our purified proteins. We will calculate both the K_D and the K_i values. Competition experiments (K_i) will be preformed by pipetting decreasing amounts of peptide and executing a sequential dilution series.
Data from these binding studies will be used for both our loop swapped SrtA proteins and the ancestrally reconstructed SrtA proteins.

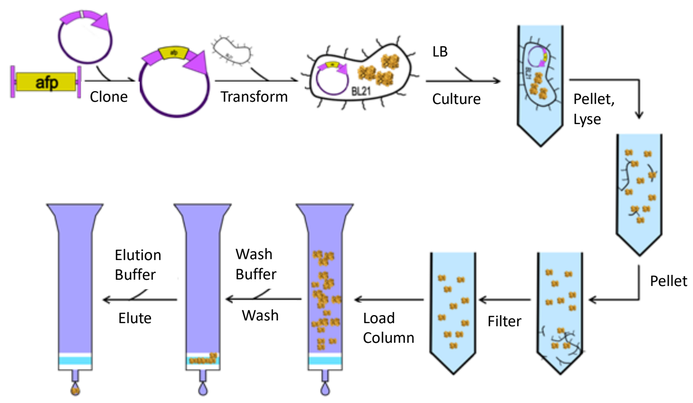

Figure 3. Protein expression and purification schematic. Base schematic obtained from University of Washington iGEM program website.[3]

Results
When purifying the two loop swapped SrtA proteins, the *Strep_swap_aureus* showed a higher monomer to dimer ratio without the refolding procedure being utilized. Isolation and purification of the resulting monomer peak yielded a conc. of 10.18mg/ml. The *Staph_swap_pneum* showed a higher monomer to dimer ratio, which was to be expected as a refolding procedure was utilized. Isolation and purification yielded a conc. of 14.17mg/ml. Both of these purified proteins were quantified using mass spectrometry (MS) to confirm their identities. Purified proteins will eventually have binding affinity and fluorescent assay experiments performed in order to measure the effects of this loop swap, these will then be compared to the $SrtA_{Strep}$ and $SrtA_{Staph}$ complexes. We hypothesize that the selectivity of Staph_swap_pneum will be more similar to S. pneumoniae, and vice versa for Strep_swap_aureus. The overall implications of these modifications on the effectiveness of sortase A overall have still yet to be quantified but the current research that has been preformed helps lay a strong framework for further exploration.

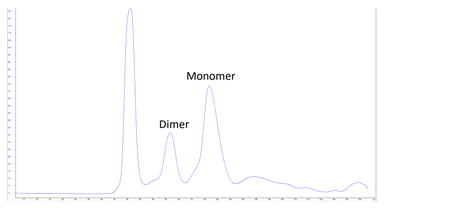

Figure 4. FPLC chromatogram for SrtA Strep_swap_aureus off SEC. Measured in mAu.

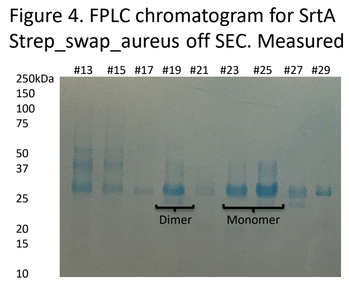

Figure 5. Tricine gel of loop swapped SrtA Strep_swap_aureus off SEC.

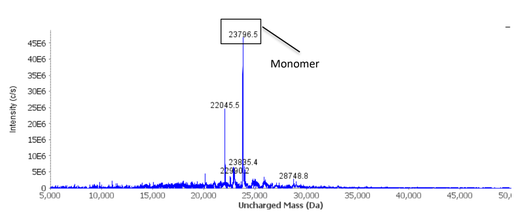

Figure 6. MS analysis of loop swapped SrtA Strep_swap_aureus.

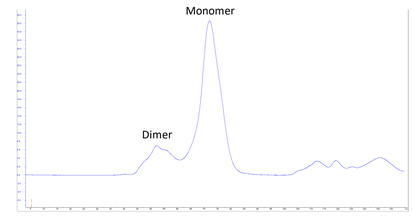

Figure 7. FPLC chromatogram for SrtA Staph_swap_pneum off SEC. Measured in mAu.

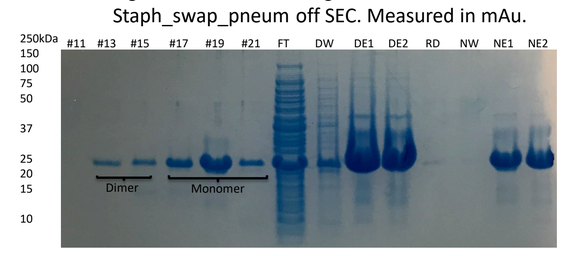

Figure 8. Tricine gel of loop swapped SrtA Staph_swap_pneum off SEC column.

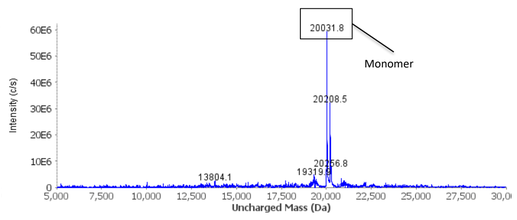

Figure 9. MS analysis of loop swapped SrtA Staph_swap_pneum.

References
[1] Antos, J. M., Truttmann, M. C., & Ploegh, H. L. (2016). Recent advances in sortase-catalyzed ligation methodology. *Current Opinion in Structural Biology,38*, 111-118. doi:10.1016/j.sbi.2016.05.021
[2] Nikghalb, K. D., Horvath, N. M., Prelesnik, J. L., Banks, O. G., Filipov, P. A., Row, R. D.. Antos, J. M. (2017). Expanding the Scope of Sortase-Mediated Ligations by Using Sortase Homologues. *ChemBioChem,19*(2), 185-195. doi:10.1002/cbic.201700517
[3] UW iGEM. (n.d.). Ideal Protein Purification. Retrieved from https://2009.igem.org/Team:Washington/Project[4]
[5] Misra, A., Biswas, T., Das S., Marathe, U., Roy, R.P., Ramakumar, S. (2015). PDB ID: 4O8L.
[6] Zong, Y., Bice, T.W., Ton-That, H., Schneewind, O., Narayana, S.V.. (2004). PDB ID: 1T2P.

Contact
Sarah Struyvenberg:
struyvs@wwu.edu
Jeanine Amacher (PI):
amachej@wwu.edu
John Antos (PI):
antosj@wwu.edu
Jordan Valgardson:
valgarj@wwu.edu