ABSTRACT

Molecular changes in *Staphylococcus aureus* fem (factors essential for methicillin resistance) mutants, analyzed via Mass Spectrometry

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As *Staphylococcus aureus* is becoming increasingly resistant to beta-lactam antibiotics, it is important to identify the mechanism of resistance on a molecular level. This study seeks to explore the molecular changes in cross-linking, cross-linking efficiency, alanylation, and O-acetylation of the *S. aureus* peptidoglycan in fem (factors essential to methicillin resistance) mutants which control glycine bridge linkage. Three different samples, BB255, UK-17, and UT34-2 were accessed by mass spectrometry, which is a technique used to measure the different molecules within the sample. The data was analyzed for changes in the cell wall via a program created by a Baylor graduate student within MassLynx. Results indicated that femB with three glycine linkages has a greater crosslinking efficiency than femA. Alanylation was similar for both mutants, and O-acetylation was slightly more prominent in femB. These results are consistent with previous experiments that used nuclear magnetic resonance techniques. Triglycine bridges of *S. aureus* peptidoglycan seem to yield more resistance to methicillin antibiotics than monoglycine bridges do.

APPROVED BY DIRECTOR OF HONORS THESIS

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Dr. Elizabeth Corey, Director

DATE:_____

MOLECULAR CHANGES IN *STAPHYLOCOCCUS AUREUS* FEM (FACTORS ESSENTIAL FOR METHICILLIN RESISTANCE) MUTANTS, ANALYZED VIA MASS SPECTROMETRY

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CHAPTER ONE

Introduction

Staphylococcus aureus

Staphylococcus aureus is a gram-positive bacterium which most frequently causes skin infections due to open wounds or post-operative surgeries (Deurenberg & Stobberingh, 2008). Although the discovery of penicillin was an effective treatment option, since then, S. aureus has acquired resistance to these beta-lactam antibiotics, prompting structure modifications like methicillin and oxacillin. The mechanism of resistance is initiated by a group of penicillin-binding proteins called PBP2a which can compensate for the cross-linking functions of PBP when it is hindered by beta-lactam antibiotics. The mecA gene codes for the PBP2a gene (Lowy, 1998).

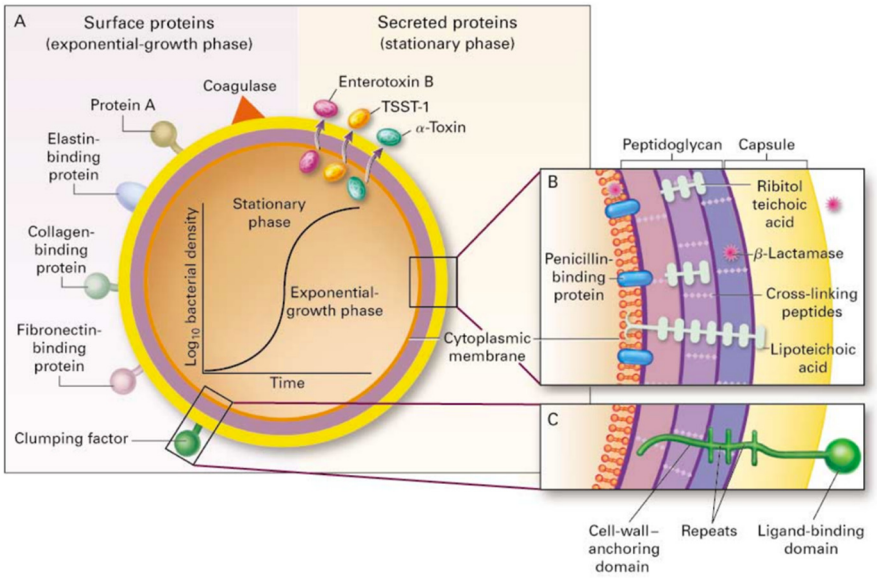
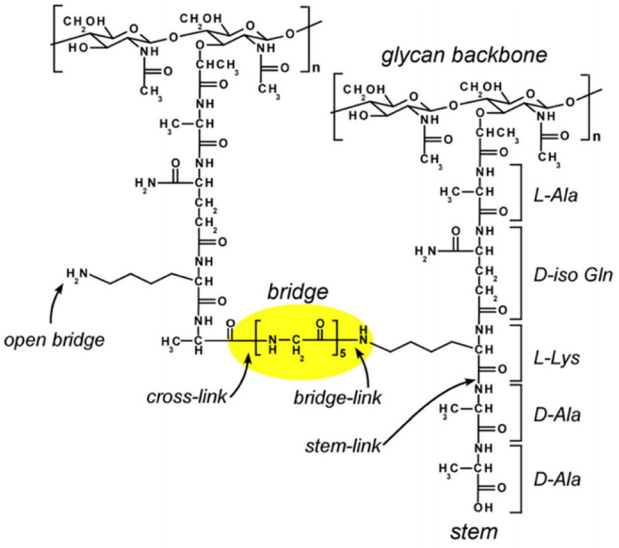


Figure 1: Cell Wall Components of Gram-Positive Bacteria

Peptidoglycan is involved in cell division and growth, protection, and attachment with other proteins or acids. One monomer of peptidoglycan consists of two sugars and a chain of amino acids. The two sugars are B-(1,4) linked N-acetylglucosamine and N-acetylmuramic acid which alternate to form the elongated cell wall structure shown in Figure 2. These subunits may be crosslinked through interactions between the amino acids of each peptide chain. The S. aureus peptidoglycan amino acids, connected to the muramic acid, are L-alanine, D-isoglutamate, L-lysine, and a pair of D-alanine. Penicillin mimics the D-ala D-ala site at the terminus and irreversibly acylates the active site of the cell wall transpeptidase. Thus, penicillin and other beta-lactams such as methicillin, explored in this study, hinder crosslinking and attempt to kill the bacteria by competitive inhibition (Yocum, Rasmussen, & Strominger, 1980).



Gram-Positive	Bridge Structure
S. aureus	Gly-Gly-Gly-Gly-Gly
FemB mutant	Gly-Gly-Gly
FemA mutant	Gly
FemX mutant	No Bridge
E. faecium	D-Asx

Figure 2: Structure of Peptidoglycan(Vollmer, Blanot, & De Pedro, 2008)

There are a group of methicillin resistant *Staphylococcus aureus* genes called fem genes, factors essential for the expression of methicillin resistance (Henze, Sidow, Wecke, Labischinski, & Berger-Bächi, 1993). It has been shown that inactivation of these genes seems to increase the susceptibility of MRSA to beta-lactams such as methicillin. The strains of S. aureus used are UK17, UT34-2, and BB255 to represent inactivation of femA, femB, and the parent strain respectively.

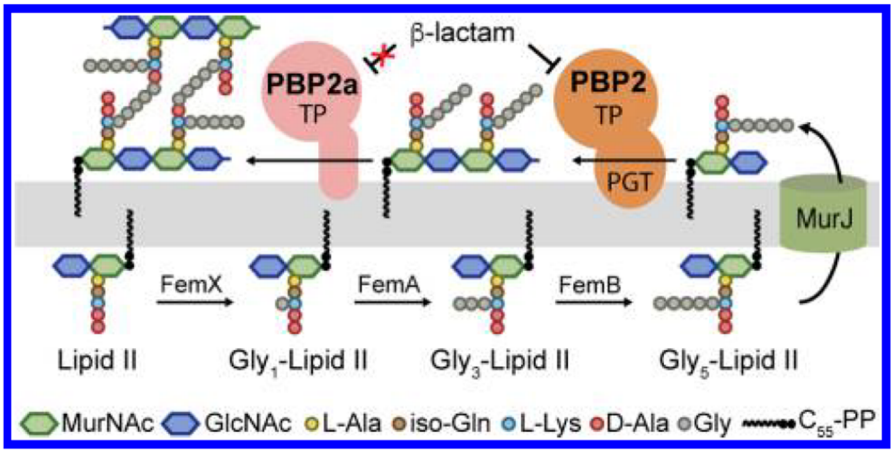


Figure 3: Function of the factors essential for methicilin resistance

The process of cell wall synthesis begins with Lipid II which is a peptidoglycan precursor. Then, the femX gene adds the first glycine to L-Lysine on the peptide chain, followed by an addition of two glycines via femA. Finally, the pentaglycine is completed with femB. Whereas femX is essential for cell survival, femA and femB are not necessary (Srisuknimit, Qiao, Schaefer, Kahne, & Walker, 2017). Although, femA and femB increase the chance of cell survival.

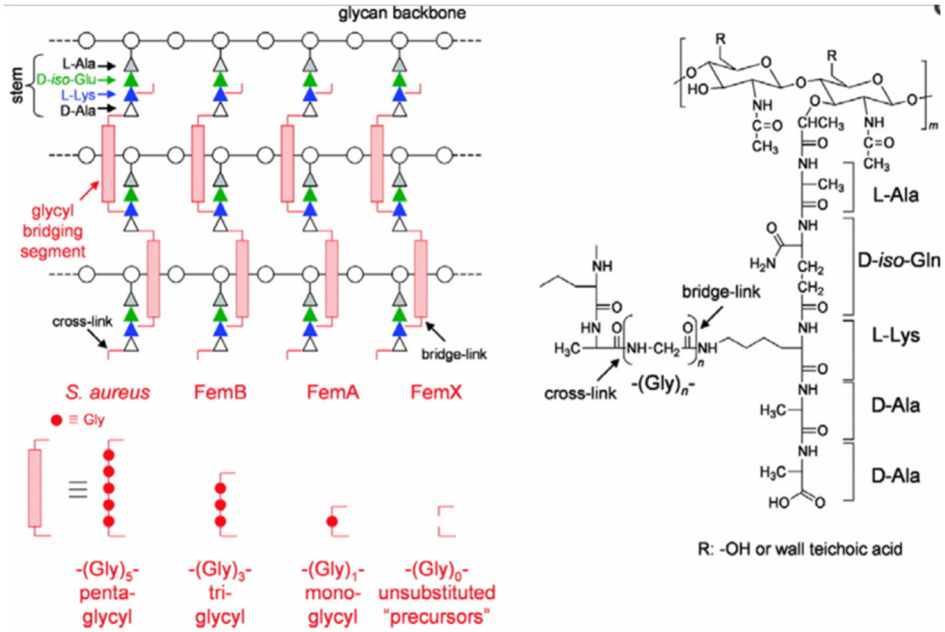


Figure 4: Fem Mutants of S. aureus(Sharif, Kim, Labischinski, & Schaefer, 2009)

Bacteria including *S. aureus* have gradually acquired resistance from antibiotics such as methicillin. The resistance is in part due to structural changes that allow for continued crosslinking. Whereas the parent S. aureus strand contains 5 glycines at the glycyl bridging segment, the femB mutant has 3 glycines, and the femA mutant has 1 glycine as shown in Figure 2. Though the mutants are characterized by their specific shortened bridge links, they also may contain a few other varying glycine bridge lengths (Sharif, Kim, Labischinski, Chen, & Schaefer, 2013). It is hypothesized that the glycine bridge link is closely correlated with crosslinking since it determines the length of the bridge. Studies have shown that inactivation of femA and femB yields changes in methicillin resistance. This study uses mass spectrometry to understand the specific structural changes in cross linking efficiency and other modifications.

The modifications analyzed using mass spectrometry are cross-linking, cross linking efficiency, alanylation, and O-acetylation state.

O-acetylation has proven to be largely related to pathogenesis. The addition of an acetyl group to the sixth carbon of the MurNAc sugar by O-acetyltransferases is a substantial determinant of lysosome resistance. Lysosome are a part of the innate immune system which serves as the first line of defense against pathogens and bacteria, hydrolyzing peptidoglycan to rupture the cell. In Figure 3, O-acetylated peptidoglycan is sterically hindered which weakens the affinity of lysosome to that part of the cell wall (Moynihan & Clarke, 2011). Pharmaceutically, the O-acetylated site is a potential target for bacterial treatments.

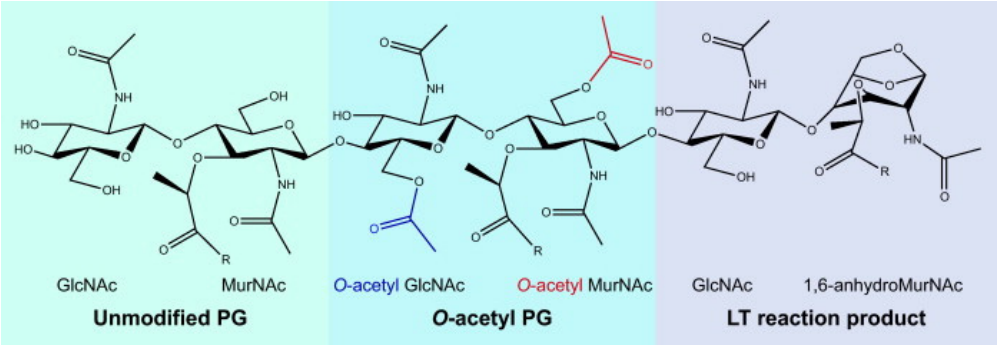


Figure 5: Peptidoglycan O-acetylation Structure

Cross-linking occurs between the L-Lysine amino acid of the peptide chain and between the two D-Alanine amino acids via transpeptidases. The bridge link consists of either one, three, or five glycine bonds.

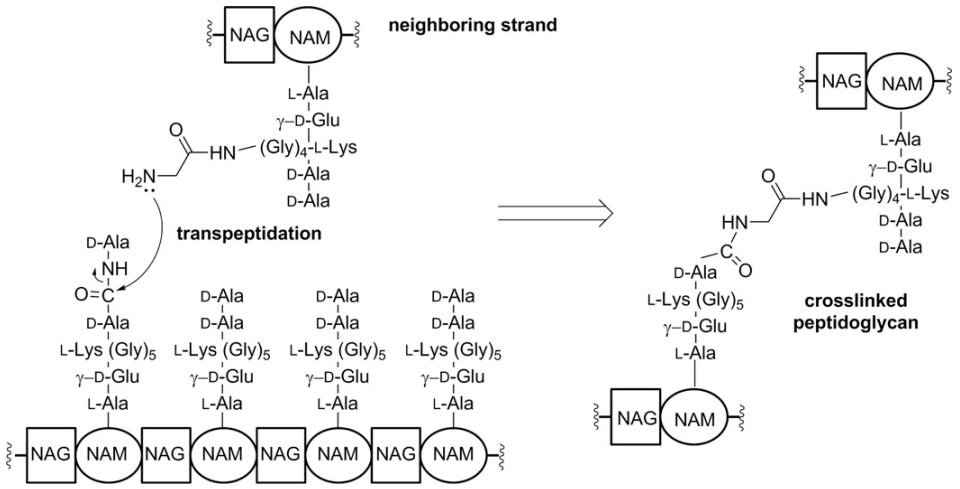


Figure 6: Transpeptidation

In Figure 5, when the peptidoglycan is crosslinked via transpeptidation, one terminal D-alanine is removed, creating a tetrapeptide instead of a pentapeptide. Pentapeptides consist of terminal D-Ala D-Ala, tetrapeptides consist of a terminal D-Ala, and tripeptides do not have D-Ala at all. D-alanine carboxypeptidases are responsible for cleaving terminal D-Ala (Izaki, Matsuhashi, & Strominger, 1968). Inhibition of D-Ala carboxypeptidase by beta-lactam antibiotics decreases crosslinking, thereby, leading to cell wall degradation. It should be noted that alanylation of the peptide is different from gene D-alanylation of teichoic acids which is controlled by a gene called dltA (Tabuchi et al., 2010).

Hypothesis

The goal of this study is to observe the structural changes in the peptidoglycan of mutant forms of methicillin-resistant staphylococcus aureus. Considering the purposes of the modifications in the cell wall of *S. aureus*, femA (UK17) should exhibit less crosslinking efficiency as well as less oligomers than femB (UT34-2). Furthermroe, femA should have less O-acetylation and less pentapeptides than femB.

CHAPTER TWO

Materials and Methods

Bacterial Growth

Bacterial growth began by inoculating 10 µl of BB255, UK-17, and UT34-2 with 1 ml of tryptic soy broth at 37°C at 180 rpm for about 12 hours. 150 µl of the overnight samples are inoculated with 15 mL of TSB under the same conditions as the previous inoculation. At a wavelength of 600 nm, the optical density of the samples was as follows:

	3 hours	After 20 min	After another 20 min
BB255	0.393	0.601	0.725
UK-17	0.257	0.424	0.540

Figure 7: Optical Density

12 mL were stored in -80°C overnight for treatment.

Pelleting and Sterilization of Bacteria

The frozen stock solutions are melted in hot water. The melted samples are spun down and re-suspended into 1 mL of phosphate-buffered saline. The re-suspended samples were boiled for 15 minutes.

Disruption of Bacterial Cell Wall

After being cooled, the samples were distributed into microcentrifuge tubes, each of which contained 0.5 mm glass beads filled up halfway. Agitation was performed using the Disruptor Genie Scientific Industries in order to purify the peptidoglycan. The

microcentrifuge tubes were shaken for 1 minute, followed by a rest period of 1 minute, and repeated the cycle six times. Then the samples, including the beads, are all combined using PBS. Using the Steriflip 20 μm nylon net vacuum filter system, the sample is separated from the beads.

Cell Wall Isolation

Next, the samples were re-suspended in 500 μl of PBS and added in the beads filter. The samples were brought up to 1 mL more with PBS. This was repeated four times with two pulses with 20 seconds between each pulse and two minutes of regular cool down between each set. The beads were allowed to settle down for one minute before the liquid portion is pipetted and spun down again. Since the UK-17 sample did not show any visible pellet, it was discarded. The supernatant was discarded and 1 mL of 2% SDS was added. The samples were boiled for 30 minutes. Thereafter, the samples were washed with 1 mL of deionized water six times. The BB255 sample was re-suspended in 1 mL of 50 mM Tris-HCl pH 8.0 and 10 μl of 10 μg/μl DNase I was added. The sample was mixed and placed in 37°C at 180 rpm.

The following day, 3 μl of 50 μg/μl of trypsin in PBS is added. The sample was placed back in 37°C at 180 rpm.

To 16 mL of TSB, 160 μl of overnight culture of UK-17 and UT-34 is added and placed back in the inoculator. It is observed after about four hours that UK-17 and UT-34 had absorbances of 0.631 and 0.722, respectively, at 600 nm. The 12 ml samples were spun down for three minutes. The supernatant was taken out and discarded, and 80 ul of

PBS was added to each of the tubes. The pellet was re-suspended and collected in a centrifuge tube to be stored in -80°C overnight for sample preparation.

The BB255 sample is lyophilized in a 15 ml Falcon tube.

Cell Wall Digestion

The samples were re-suspended in 1 mL of 20 mM Tris, pH 8.0 HPLC grade. The mutanolysin in 250 µl of the boron. Furthermore, 20 µl of mutanolysin solution is added to each Falcon tube with the sample, vortexed, and stored at room temperature. After 24 hours, another 20 µl of mutanolysin solution is added to the samples.

Filtration

The samples were spun down through a 0.45 um and 30 kD filter and stored in -80°C.

Sodium Borohydride Reduction

To the lyophilized samples, 20 µl of sodium borohydride at concentration 0.5 mg/µl in borate buffer pH 9.0 with a final concentration of 10 mg/µl is added. The samples were then suspended in 1 mL of 0.5 M borate buffer of pH 9.0. Mixing was performed by tilting the Falcon tubes and incubation for 30 minutes rather than by vortexing.

After 30 minutes, 120 µl of phosphoric acid was added to the samples which underwent vortexing and frozen at -80°C for lyophilization.

Sample Preparation

After lyophilization the final sample weight of BB255, UK-17, and UT-34 were 6.910 g, 6.968 g, and 6.884 g respectively. To the samples, 1 mL of 20 mM Tris-HCl, pH

8.0 HPLC grade water is added. 500 µl of each dissolved sample is filtered through a 0.45 ⌈l filter. From the filtered sample, 200 µl is transferred to the MS vials and stored at 4°C in preparation for Liquid Chromatography-Mass Spectrometry runs.

Data Analysis

The results from mass spectrometry were analyzed via MassLynx. MATLAB (MathWorks) was also used in order to calculate the desired matches of peptidoglycan masses with specifications in the O-acetylation state, oligomeric length, and alanylation.

CHAPTER THREE

Results

BB255

c	ala	OAc	NDc	z	mass	Avg subunit
2	2	0	0	3	3580.647	17565
0	0	0	0	2	1070.508	10473
2	2	1	0	3	3622.657	8410
1	2	0	0	3	2417.116	5608
2	2	2	0	3	3664.668	4895
0	1	0	0	2	1141.545	3776
0	0	0	0	1	1111.511	3401
1	2	1	0	3	2459.127	1936
1	2	0	0	2	2417.116	1928
1	1	0	0	2	2305.076	1899
1	2	1	0	2	2459.127	1117
0	1	1	0	1	1224.559	400
1	1	0	0	3	2346.079	367
1	1	0	0	2	2346.079	277
2	2	0	0	3	3539.644	210
1	1	2	0	3	2430.1	102

Figure 8: Heatmap of Crosslinking, Alanylation, O-acetylation, Charge, Mass, and Average Subunits for BB255

UK17						
c	Ala	OAc	NDc	z	mass	Avg subunit
0	2	0	0	1	1025.4991	201240
2	2	0	0	3	2896.3873	79814
2	2	1	0	3	2938.3979	49409
3	2	0	0	4	3831.8314	28251
0	0	1	0	1	925.4354	28105
1	2	0	0	2	1960.9432	24326
3	2	1	0	4	3873.8420	24226
2	2	2	0	3	2980.4084	18530
2	2	0	-1	3	2855.3840	16948
1	2	1	0	2	2002.9537	14796
2	1	0	0	3	2825.3502	14395
2	1	1	0	3	2867.3607	14090
0	1	1	0	1	996.4725	10210
3	2	0	-1	4	3790.8281	7097
2	0	1	0	3	2796.3236	7008
1	1	0	0	2	1889.9061	6255
3	1	1	0	4	3802.8049	5401
3	1	0	0	4	3760.7943	4429
1	2	0	0	3	1960.9432	3981
2	1	0	-1	3	2784.3469	3268
3	2	2	0	4	3915.8525	3258
1	2	0	-1	2	1919.9399	2847
3	0	1	0	4	3731.7677	2510
1	2	1	0	3	2002.9537	2298
1	0	1	0	2	1860.8795	2022
1	1	0	-1	2	1848.9028	777
1	2	0	-1	3	1919.9399	685
1	0	0	0	2	1818.8689	497
1	0	1	0	3	1860.8795	313
1	1	0	0	3	1889.9061	304

Figure 9: Heatmap of Crosslinking, Alanylation, O-acetylation, Charge, Mass, and Average Subunits for UK-17

JT34-2

c	Ala	OAc	NDc	z	mass	Avg subunit
2	2	0	0	3	3238.5181	32600
3	2	0	0	4	4288.0058	29708
2	2	1	0	3	3280.5287	25775
3	2	1	0	4	4330.0164	21147
2	2	0	-1	3	3197.5148	11138
3	0	2	0	4	4229.9527	8316
3	2	0	-1	4	4247.0025	8103
1	2	0	0	2	2189.0304	7622
2	1	0	0	3	3167.4810	7538
1	2	0	0	3	2189.0304	7181
2	2	2	0	3	3322.5392	7009
0	1	1	0	1	1110.5161	6669
0	2	0	0	1	1139.5427	5692
3	1	0	0	4	4216.9687	4240
1	2	1	0	3	2231.0409	4068
2	0	1	0	3	3138.4544	3918
3	0	1	0	4	4187.9421	3523
1	2	1	0	2	2231.0409	3197
0	1	0	0	1	1068.5056	2968
1	1	0	0	2	2117.9933	1739
1	2	0	-1	3	2148.0271	1559
1	1	0	0	3	2117.9933	1068
1	0	1	0	2	2088.9667	892
1	2	0	-1	2	2148.0271	764
1	1	0	-1	2	2076.9900	607
1	1	1	0	3	2160.0038	279
1	1	0	-1	3	2076.9900	267

Figure 10: Crosslinking, Alanylation, O-acetylation, Charge, Mass, and Average Subunits for UT34-2

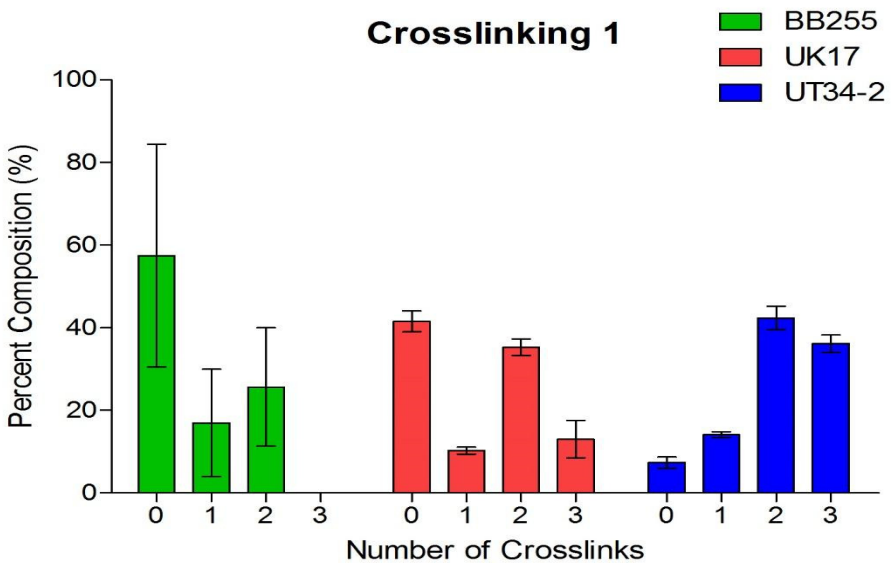


Figure 11: Number of Crosslinks for fem mutants with 0, 1, 2, and 3 representing monomers, dimers, trimers, and tetramers respectively

	Avg	SD	95% CI
BB255			
0	57.4166	10.8488	19.9307
1	16.9280	5.2256	9.6002
2	25.6553	5.7645	10.5901
3	0	0	0
UK17			
0	41.5047	1.0214	1.8765
1	10.2376	0.3403	0.6253
2	35.2449	0.8136	1.4946
3	13.0128	1.8301	3.3620
UT34-2			
0	7.3913	0.5421	0.9959
1	14.0766	0.3101	0.5697
2	42.3692	1.1506	2.1137
3	36.1628	0.8385	1.5403

Figure 12: Data for Percent Composition of Monomers, Dimers, Trimers, and Tetramers for fem mutants

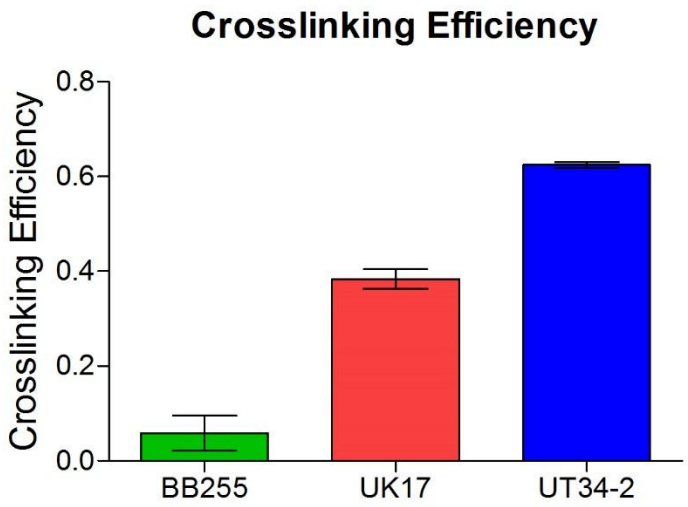


Figure 13: Crosslinking efficiency of wild-type, femA, and femB

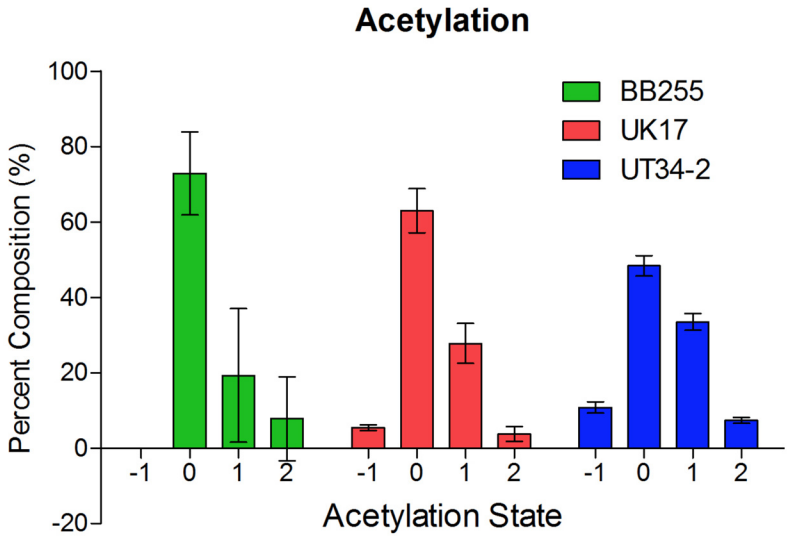


Figure 14: Acetylation State for fem mutants

	Avg	SD	95% CI
BB255			
-1	0	0	0
0	72.88341	4.44646	8.168718
1	19.28702	7.148201	13.13216
2	7.829569	4.492054	8.25248
UK17			
-1	5.479213	0.298929	0.549172
0	62.98439	2.348484	4.314467
1	27.76699	2.138009	3.927798
2	3.769407	0.788831	1.449183
UT34-2			
-1	10.78844	0.602491	1.106853
0	48.36831	1.09229	2.006677
1	33.46827	0.89826	1.65022
2	7.374984	0.325413	0.597826

Figure 15: Statistical Data for Acetylation States of fem mutants

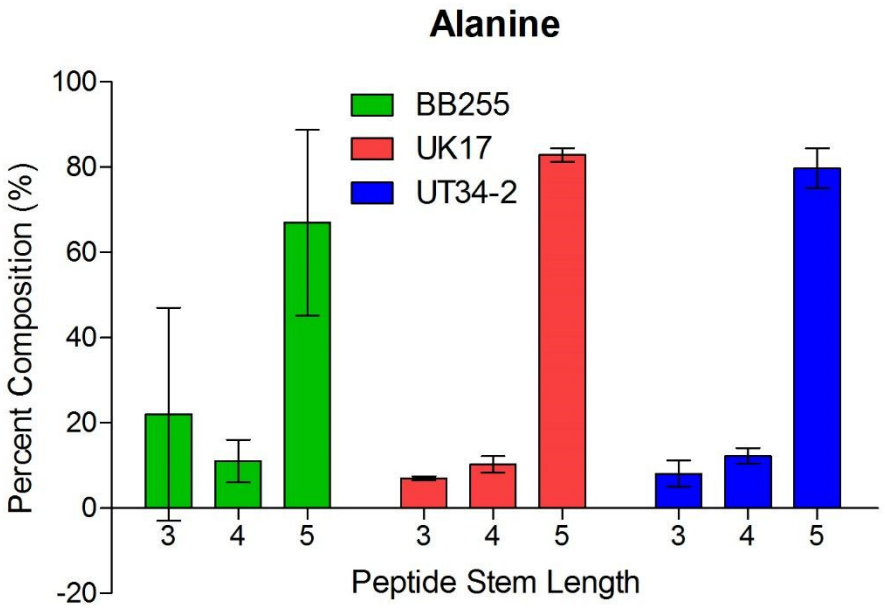


Figure 16: Changes in Alanylation for fem mutants

	Avg	SD	95% CI
BB255			
tripeptide	22.04636	10.04913	18.46154
tetrapeptide	11.02017	2.022071	3.714804
pentapeptide	66.93347	8.7613	16.09563
UK17			
tripeptide	7.008955	0.185245	0.34032
tetrapeptide	10.24043	0.789385	1.450202
pentapeptide	82.75061	0.633747	1.164275
UT34-2			
tripeptide	8.066507	1.233868	2.266774
tetrapeptide	12.24354	0.735091	1.350457
pentapeptide	79.68996	1.891843	3.475559

Figure 17: Table of Alanylation Changes of fem mutants

CHAPTER FOUR

Discussion and Conclusion

Crosslinking Efficiency

The average cross linking efficiency of UK17 is 38.4% compared to that of UT34-2 which is 62.4%. A study on the architecture of peptidoglycan using NMR revealed that although the bridge link percentage was precise for pentaglycine, triglycine, and monoglycine structures, the crosslinking percentage for femA was only a third of that of femB and the wild type (Kim, Chang, & Singh, 2015). In femB, 70% crosslinking was observed, whereas in femA, 50% crosslinking was observed. Assuming the results from the NMR study are reasonable, the percent error for the mass spectrometry data is 23.2% for femA and 10.9% for femB. Thus, these results are consistent with the observation that the mutant with the monoglycine peptidoglycan bridge structure demonstrates a lower value for crosslinking efficiency than that of a triglycine peptidoglycan bridge structure.

Organisms	PG-bridge structure	Bridge-links [%]	Cross-links [%]
S. aureus (ATCC 6538P)	Gly-Gly-Gly-Gly-Gly	85	67
Wild type *S. aureus* (BB255)	Gly-Gly-Gly-Gly-Gly	94	75
FemB (UT34-2)	Gly-Gly-Gly	92	70
FemA (UK 17)	Gly	91	50
E. faecium (ATCC 49624)	D-Asp	61	51

Figure 18: NMR data of Crosslinking for Variations of *S. Aureus*

Oligomeric Crosslinking

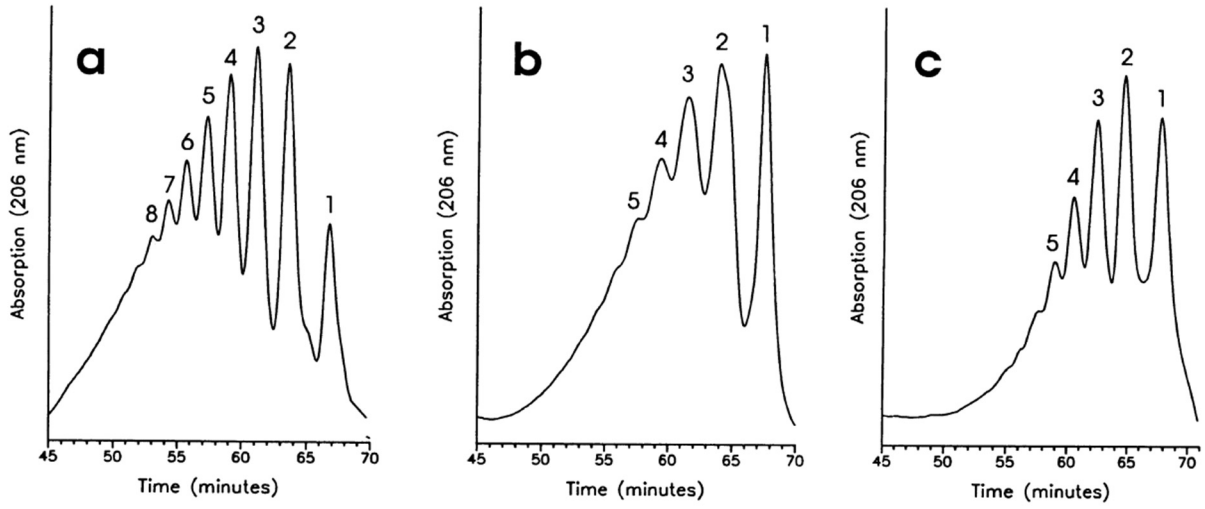


Figure 19: HPLC data of Oligomers Observed in (a) parent strain (b) femA (c) femB

As for the oligomers of crosslinking, UK-17 had mostly monomers at 41.5%, followed by trimers at 35.2%. In contrast, trimers dominated in UT34-2 at 42.4% and 36.2% tetramers. UK-17, which is the femA strain consisting of one glycine bridge, exhibited much less transpeptidase activity compared to UT34-2, femB with the triglycine bridge. These findings are consistent with a recent study which found that PBP2a is capable of crosslinking femB, but not femA (Srisuknimit et al., 2017). Based on Figure 20, the activity of PBP2a suggests that femA is a better target for beta-lactam potentiators than femB. There have been studies that concluded that a reduction in crosslinking yields a reduction in the stiffness of the cell wall (Loskill et al., 2014).

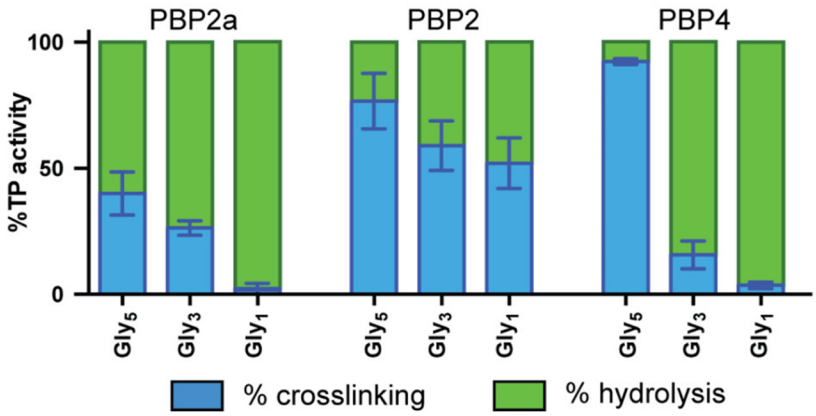


Figure 20: Percent Transpeptidase Activity Among Peptidoglycan Binding Proteins

Pentapeptides were the most prevalent in UK-17 and UT34-2, yielding 82.8% and 79.7%. There were few tetrapeptides detected: 10.2% for UK-17, 12.2% for UT34-2. There were even fewer tripeptides detected: 7.0% for UK-17, 8.1% for UT34-2.

Acetylation

The percent composition of the MurNac sugar with an O-Acetylation state of zero for BB255, UK17, and UT34-2 is 72.9%, 63.0% and 48.4% respectively. The combined acetylation state of 1 and 2 for UK17 is 31.4% while that of UT34-2 is 40.7%. O-acetylation of the MurNac sugar has been observed to be a determinant of lysosome resistance which strengthens the peptidoglycan of S. aureus and other bacteria. Thus, it was expected for UT34-2 to show more O-acetylation than UK17 since it correlates with the greater percentage in crosslinking efficiency of femB compared to UK17.

Alanylation

The degree of alanylation for UK17 and UT34-2 are similar. The percentage of pentapeptides for BB255, UK17, and UT34-2 is 66.9%, 82.8%, and 79.7% respectively.

Sources of Error

The most prominent source of error was the low peak intensity for the BB255 spectra. This caused chromatograms to be unreliable, which is a possible reason for the large uncertainties seen in the standard deviations of these values.

Application

Understanding the different characteristics of S. aureus growth depending on the length of the glycine bridge enables researchers to develop pharmaceutical drugs that target specific mechanisms. Performing this experiment in vitro lessens the possibilities of confounding variables and allows the fem mutants to be observed in isolation.

Future Prospects

Although the wild type strain, BB255, produced results for crosslinking, oligomerization, acetylation, and alanylation, the results were neither consistent with previous data nor analyzable due to the small sample size. Possible sources of error may be associated with the mass spectrometer, preparation of the solution, or conditions under which the same was prepared.

Nevertheless, the data for UK-17 and UT34-2 are reliable. This study on the chemical changes to the cell wall such as crosslinking of fem mutants consisting of either three or one glycine bridge may be continued and strengthened with testing of a wild type strain. Data about the wild type would add a reference point to compare the mutant species with.

APPENDIX

MATLAB Script for PG Library Generation and Matching

The following program made via MATLAB was used for the purpose of generating possible combinations of peptidoglycan modification masses and finding matches of mass-to-charge ratios in accordance with the peptidoglycan results.

```
% fem mutants possibility matrix script
% Now with:
% m/z query matching
% glufib correction

%generates 3 ppm differences and 3 intensities

clear all
clc

% Initializing the matrix
Modification_variables = 20;
Number_of_variations = 3000000;
Library(Number_of_variations,Modification_variables) = 0;

% Variable parameters
crosslink_minimum = 1;
crosslink_maximum = 1;
sugar_missing_minimum = 0;
alanine_minimum = 0;
lactate_minimum = 0;
O_acetylation_minimum = 0;
N_deacetylation_minimum = 0;
label_minimum = 0;
bridge_missing_minimum = 0;
query_charge_limit = 8;
%place values in proper column in library
starting_charge_column = 9;


% Mass parameters
mass_proton = 1.0072764668;
start_mass = 1109.4956885120;
cross_link_mass = 1161.5149611613;
alanine_mass = 71.0371137878;
lactate_addition_mass = 0.9840155848;
O_Ac_mass = 42.0105646863;
N_DAc_mass = 41.0032882341;
```

```
sugar_missing_mass = 477.1725979864;
label_medium = 8.0502139672;
label_heavy = 17.0706895263;
FemB_missing_mass = 114.0429;
FemA_missing_mass = 228.0865;
bridge_missing_mass = 287.1229686822;



% Looping crosslinks
for c = crosslink_minimum:crosslink_maximum

    % Looping label
    % for light, label=0
    % for medium, label=1
    % for heavy, label=2
    label_maximum = 2;

    for label = label_minimum:label_maximum;

        if label == 0
            difference = 0;
            number_enrichment = 0;
        elseif label == 1
            difference = label_medium;
            number_enrichment = 8.5;
        elseif label == 2
            difference = label_heavy;
            number_enrichment = 17;
        end

        % Looping missing sugars
        sugar_missing_maximum = 1 + c;
        % sugar_missing_maximum = 0;
        for sugar_missing =
sugar_missing_minimum:sugar_missing_maximum

            % Looping alanine
            alanine_maximum = 2;
            for ala = alanine_minimum:alanine_maximum;

                % Looping lactate
                %lactate_maximum = floor(ala/2);
                lactate_maximum = 0;
                if lactate_maximum < 0
                    lactate_maximum = 0;
                end
                for lactate = lactate_minimum:lactate_maximum

                    % Looping O-Acetylation
                    % O_acetylation_maximum = c + 1 - sugar_missing;
                    O_acetylation_maximum = 0;
                    for O_Ac =
O_acetylation_minimum:O_acetylation_maximum
```

```
                        % Looping N-Deacetylation
                        % N_deacetylation_maximum = c*1 + 1;
                        N_deacetylation_maximum = 0;
                        for N_DAc =
N_deacetylation_minimum:N_deacetylation_maximum

                            % Looping bridge/Fem missing
                            bridge_missing_maximum = 0;
                            for bridge_missing =
bridge_missing_minimum:bridge_missing_maximum

                                % Bridge/Fem missing counter
                                % 0 = intact bridge, 1 = FemB (2 Gly
                                % missing), 2 = FemA (4 Gly
missing), 3 =
                                % all 5 Gly missing
                                if bridge_missing == 0
                                    bridge_subtract = 0;
                                elseif bridge_missing == 1
                                    bridge_subtract =
FemB_missing_mass * (c + 1);
                                elseif bridge_missing == 2
                                    bridge_subtract =
FemA_missing_mass * (c + 1);
                                elseif bridge_missing == 3
                                    bridge_subtract =
bridge_missing_mass;
                                end

                                Library_count = Library_count + 1;
                                Library(Library_count,1) = c;
                                Library(Library_count,2) = ala;
                                Library(Library_count,3) = O_Ac;
                                Library(Library_count,4) =
sugar_missing*(-1);
                                Library(Library_count,5) = label;
                                Library(Library_count,6) =
number_enrichment * (c + 1);
                                exact_mass = start_mass
+(c*cross_link_mass)+(ala*alanine_mass)+(O_Ac*O_Ac_mass) + (N_DAc*(-
1)*N_DAc_mass)+ ((sugar_missing*(-1))*sugar_missing_mass) + ((c +
1)*(difference)) + (lactate*lactate_addition_mass) + ((-
1)*bridge_subtract);
                                Library(Library_count,7) =
exact_mass;
                                initial_charge = N_DAc +
sugar_missing;
                                if initial_charge == 0
                                    initial_charge = 1;
                                end
                                Library(Library_count,8) =
initial_charge;
```

```
                                Library(Library_count,18) = N_DAc*(-
1);
                                Library(Library_count,19) = lactate;
                                Library (Library_count,20) =
bridge_missing;

                                % Filling out the m/z columns
                                for charge_count =
0:query_charge_limit
                                    Library(Library_count,
(starting_charge_column + charge_count)) = ((exact_mass +
charge_count * mass_proton) / (initial_charge + charge_count));
                                end

                            end

                        end

                    end

                end

            end

        end

    end

end


% Reading the combinatorically generated library
target_index_size = Library_count;
target = Library(1:target_index_size,:);


% Correcting Library for glufib

%defining glufib values
glufib_theoretical = 785.8457;
glufib_observed = 785.8629;

%array of zeros
target_glufib_array = zeros(target_index_size,18);

%adding values to array
target_glufib_array(:,1:8) = target(:,1:8);
target_glufib_array(:,9:17) = (target(:,9:17)) *
(glufib_observed/glufib_theoretical);
target_glufib_array(:,18:20) = target(:,18:20);

for target_glufib_rows = 1:target_index_size

    for target_glufib_columns = 9:17
```

```
        if target_glufib_array(target_glufib_rows,
target_glufib_columns) >= 2000
            target_glufib_array(target_glufib_rows,
target_glufib_columns) = 0;
        end

    end

end


%Generating distribution array (peak heights of light species)

% Initializing the chemical formula variables (creating 2 separate
arrays of zeros as
% placeholders)
chemical_formula = zeros (target_index_size,5);
peak_heights = zeros (target_index_size,13);

% Loop for defining chemical formula and determining peak
distribution for
% each chemical formula
for entry_number = 1:target_index_size

    %Caculating number of C,H,N,O,S for each library entry
    chemical_formula(entry_number,1) = 39 +
target(entry_number,1)*42 + target(entry_number,2)*3 +
target(entry_number,3)*2 + target(entry_number,4)*(8) +
target(entry_number,18)*2 + target(entry_number,19)*(0) +
target(entry_number,20)*(-10);
    chemical_formula(entry_number,2) = 70 +
target(entry_number,1)*72 + target(entry_number,2)*5 +
target(entry_number,3)*2 + target(entry_number,4)*(13) +
target(entry_number,18)*1 + target(entry_number,19)*(-1) +
target(entry_number,20)*(-17);
    chemical_formula(entry_number,3) = 9 + target(entry_number,1)*10
+ target(entry_number,2)*1 + target(entry_number,3)*0 +
target(entry_number,4)*(1) + target(entry_number,18)*0 + target
(entry_number,19)*(-1) + target(entry_number,20)*(-5);
    chemical_formula(entry_number,4) = 19 +
target(entry_number,1)*19 + target(entry_number,2)*1 +
target(entry_number,3)*1 + target(entry_number,4)*(5) +
target(entry_number,18)*1 + target (entry_number,19)*(1) +
target(entry_number,20)*(-5);
    distribution = isotopicdist([chemical_formula(entry_number,1)
chemical_formula(entry_number,2) chemical_formula(entry_number,3)
chemical_formula(entry_number,4) chemical_formula(entry_number,5)]);

    %Putting data into array (rows are library entries, columns are
    %relative peak heights of up to ten peaks)
    distribution_length = length (distribution);
    if distribution_length >= 10
        distribution_length = 10;
```

```
    end
    for distribution_number = 1:distribution_length
        peak_heights (entry_number,distribution_number) =
distribution (distribution_number,2);
    end

end


%initializing binomial distribution array (zeros as placeholders)
binomial_dist_array = zeros (4,target_index_size);
percent_enrichment_medium = 0.980;
percent_enrichment_heavy = 0.980;

for binomial_dist_count = 1:target_index_size

    if target(binomial_dist_count,5) > 0
        %specifying medium or heavy
        if target(binomial_dist_count,5) == 1
            temporary_percent_enrichment =
percent_enrichment_medium;
        else
            temporary_percent_enrichment = percent_enrichment_heavy;
        end

        %generating binomial distribution array
        for binomial_dist_peaks = 0:3
            %calculating binomial coefficient
            binomial_coefficient =
factorial(target(binomial_dist_count,6))/(factorial(binomial_dist_pe
aks)*(factorial(target(binomial_dist_count,6)-
binomial_dist_peaks)));
            binomial_expression =
(temporary_percent_enrichment^(target(binomial_dist_count,6)-
binomial_dist_peaks))*((1-
temporary_percent_enrichment)^(binomial_dist_peaks));
            binomial_dist_array(binomial_dist_peaks + 1,
binomial_dist_count) = binomial_coefficient*binomial_expression;
        end

    else
            binomial_dist_array(1,binomial_dist_count) = 1;

    end

end

%initializing combined array
combined_array = zeros (target_index_size,16);

%combining distribution and binomial distribution
for library_entry = 1:target_index_size

    %initializing product array
```

```
    product_array = zeros(16,4);

    for distribution_entry = 1:10

        for binomial_entry = 1:4

            %defining each element in product array
            product_array(distribution_entry + 3,binomial_entry) =
peak_heights(library_entry,distribution_entry) *
binomial_dist_array(binomial_entry,library_entry);

        end

    end


   %putting values in combined array (sum of products)
   for rows = 1:13

       for term = 0:3

           combined_array(library_entry,rows) =
combined_array(library_entry,rows) + product_array(rows + term, 1 +
term);

       end

   end

end


%making array of correction factors
correction_factor_array = zeros(target_index_size,1);
for correction_factor_count = 1:target_index_size
    correction_factor_array(correction_factor_count,1) =
max(combined_array(correction_factor_count,:));
end


% Reading the manually picked m/z targets of interest
[mz_query] = xlsread('PG matches Fem mutants.xlsx',3,'E4:O32');
mz_query_index_size = length(mz_query);

% Defining search parameters and initializing the targets
tolerance = 50; % in ppm
match(5000,31) = 0;
match_index = 1;

% Search algorithm
for mz_query_index = 1:mz_query_index_size

    % Setting the m/z query targets
```

```
    %observed
    current_observed_mz_01 = mz_query(mz_query_index,3);
    current_observed_mz_02 = mz_query(mz_query_index,6);
    current_observed_mz_03 = mz_query(mz_query_index,9);

    %observed corrected for glufib
    current_query_mz_01 = mz_query(mz_query_index,4);
    current_query_mz_02 = mz_query(mz_query_index,7);
    current_query_mz_03 = mz_query(mz_query_index,10);

    current_query_charge = mz_query(mz_query_index,1);

    current_query_intensity_01 = mz_query(mz_query_index,5);
    current_query_intensity_02 = mz_query(mz_query_index,8);
    current_query_intensity_03 = mz_query(mz_query_index,11);


    % Moving the target from one to another while making the
comparison
    for target_index = 1:target_index_size

        % Taking the difference
        target_charge_column = current_query_charge + 8 -
(target(target_index,8) - 1);
        mz_difference_01 = current_query_mz_01 -
target(target_index,target_charge_column);
        mass_difference_01 = mz_difference_01*current_query_charge;
        ppm_difference_01 =
(mass_difference_01/target(target_index,7))*1000000;

        target_charge_column = current_query_charge + 8 -
(target(target_index,8) - 1);
        mz_difference_02 = current_query_mz_02 -
target(target_index,target_charge_column);
        mass_difference_02 = mz_difference_02*current_query_charge;
        ppm_difference_02 =
(mass_difference_02/target(target_index,7))*1000000;

        target_charge_column = current_query_charge + 8 -
(target(target_index,8) - 1);
        mz_difference_03 = current_query_mz_03 -
target(target_index,target_charge_column);
        mass_difference_03 = mz_difference_03*current_query_charge;
        ppm_difference_03 =
(mass_difference_03/target(target_index,7))*1000000;

        %Determining minimum ppm difference
        ppm_difference_array = zeros(3,1);
        ppm_difference_array(1,1) = abs(ppm_difference_01);
        ppm_difference_array(2,1) = abs(ppm_difference_02);
        ppm_difference_array(3,1) = abs(ppm_difference_03);
        ppm_difference_min = min(ppm_difference_array(1:3,1));
```

```
        % Comparing the difference and adding to the matches if
found
        if ppm_difference_min < tolerance && current_query_charge >=
target(target_index,8)
            match(match_index,1) = target_index;
            match(match_index,2) = mz_query(mz_query_index,2);
            match(match_index,3:10) = target(target_index,1:8);
            match(match_index,11) = current_query_charge;
            match(match_index,12) = current_observed_mz_01;
            match(match_index,13) = current_observed_mz_02;
            match(match_index,14) = current_observed_mz_03;
            match(match_index,15) = current_query_mz_01;
            match(match_index,16) = current_query_mz_02;
            match(match_index,17) = current_query_mz_03;
            match(match_index,18) =
target(target_index,target_charge_column);
            match(match_index,19) = ppm_difference_01;
            match(match_index,20) = ppm_difference_02;
            match(match_index,21) = ppm_difference_03;
            match(match_index,22) = current_query_intensity_01;
            match(match_index,23) = current_query_intensity_02;
            match(match_index,24) = current_query_intensity_03;
            match(match_index,25) =
correction_factor_array(target_index,1);
             %observed monoisotopic peak intensities
            match(match_index,26) = current_query_intensity_01 /
correction_factor_array(target_index,1);
            match(match_index,27) = current_query_intensity_02 /
correction_factor_array(target_index,1);
            match(match_index,28) = current_query_intensity_03 /
correction_factor_array(target_index,1);
            match(match_index,29) = target(target_index,18);
            match(match_index,30) = target(target_index,19);
            match(match_index,31) = target(target_index,20);
            match_index = match_index + 1;
        end

    end

end


% lysine incorporation

HH_HL_ratio = 2.50;
prob_H = (2 * HH_HL_ratio) / (1 + (2 * HH_HL_ratio));

% initializing corrected match array
match_corrected = zeros(match_index, 31);
match_corrected(:, 1:25) = match(1:match_index, 1:25);
match_corrected(:,29:31) = match(1:match_index, 29:31);


% adjusting all heavy and light intensities
```

```
multimer_min = crosslink_minimum + 1;
multimer_max = crosslink_maximum + 1;

for counting_multimers = multimer_min:multimer_max
    HH_per_x = (prob_H)^counting_multimers;
    LL_per_x = (1 - prob_H)^counting_multimers;

    % correcting all heavy intensities
    for match_entry = 1:match_index

        if match(match_entry, 7) == 2
            match_corrected(match_entry, 26) = match(match_entry,26)
/ HH_per_x;
            match_corrected(match_entry, 27) = match(match_entry,27)
/ HH_per_x;
            match_corrected(match_entry, 28) = match(match_entry,28)
/ HH_per_x;
        end

    end

    %correcting all light species by searching corresponding heavy
and
    %subtracting heavy contribution
    for specie_to_be_matched = 1:match_index

        for searching_light = 1: match_index

            if match(searching_light, 7) == 0

                for finding_corresponding_heavy = 1:match_index

                    if match(finding_corresponding_heavy, 7) == 2

                        for counting_c = 1:match_index

                            if match(counting_c, 3) ==
match(specie_to_be_matched ,3)

                                for counting_ala = 1:match_index

                                    if match(counting_ala, 4) ==
match(specie_to_be_matched, 4)

                                        for counting_z =
1:match_index

                                            if match(counting_z, 11)
== match(specie_to_be_matched, 11)

                                                if counting_c ==
counting_ala && counting_ala == counting_z
```

```
                                                    if
match(specie_to_be_matched,7) == 0

match_corrected(specie_to_be_matched, 26) =
match(specie_to_be_matched, 26) - (match(counting_z, 26) *
(LL_per_x/HH_per_x));

match_corrected(specie_to_be_matched, 27) =
match(specie_to_be_matched, 27) - (match(counting_z, 27) *
(LL_per_x/HH_per_x));

match_corrected(specie_to_be_matched, 28) =
match(specie_to_be_matched, 28) - (match(counting_z, 28) *
(LL_per_x/HH_per_x));
                                                    end

                                                end

% chemical formula calculation for all the matches

match_chemical_formula = zeros (match_index,5);
for formula_index = 1:match_index

    % Caculating number of C,H,N,O for each match entry
    match_chemical_formula(formula_index,1) = 39 +
match(formula_index,3)*42 + match(formula_index,4)*3 +
match(formula_index,5)*2 + match(formula_index,6)*(8) +
match(formula_index,29)*2 + match(formula_index,30)*(0) +
match(formula_index,31)*(-10);
    match_chemical_formula(formula_index,2) = 70 +
match(formula_index,3)*72 + match(formula_index,4)*5 +
match(formula_index,5)*2 + match(formula_index,6)*(13) +
match(formula_index,29)*1 + match(formula_index,30)*(-1) +
match(formula_index,31)*(-17);
    match_chemical_formula(formula_index,3) = 9 +
match(formula_index,3)*10 + match(formula_index,4)*1 +
match(formula_index,5)*0 + match(formula_index,6)*(1) +
match(formula_index,29)*0 + match(formula_index,30)*(-1) +
match(formula_index,31)*(-5);
    match_chemical_formula(formula_index,4) = 19 +
match(formula_index,3)*19 + match(formula_index,4)*1 +
match(formula_index,5)*1 + match(formula_index,6)*(5) +
match(formula_index,29)*1 + match(formula_index,30)*(1) +
match(formula_index,31)*(-5);

end
```

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