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Characterizing Bacterial Cell Wall Synthesis Through the Use of Fluorescent Stem Peptide Mimics

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CHARACTERIZING BACTERIAL CELL WALL SYNTHESIS THROUGH THE USE OF FLUORESCENT STEM PEPTIDE MIMICS

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Background

Bacteria, one of the three domains of life, consists of small, simple, single-celled prokaryotic organisms which lack membrane-bound organelles[1,2]. Although microscopic in size, bacteria are a highly diverse clade which can survive in nearly every niche across the planet. These miniscule organisms are of particular importance to humans because they are responsible for a variety of human diseases. MRSA, diptheria, anthrax, leprosy, tuberculosis, tetanus, whooping cough, and cholera are just a few of the human illnesses caused by bacteria[2-3].

A characteristic feature of bacteria is the presence of a cell wall composed of an intricate, mesh-like polymer called peptidoglycan. This molecule plays a critical role in maintaining the structural integrity of bacterial cells by helping them withstand turgor pressure and consists of sugars and peptides[4]. The sugars are arranged in long chains made of alternating N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM) residues[5]. A short four or five amino acid peptide (stem peptide) is attached to each NAM residue[5]. The sequence of the stem peptide is relatively conserved; however, some inter and intraspecies variations exist[5]. The greatest variation in the stem peptide architecture lies in the third position with one group of organisms having L-Lysine in the position and the other having the noncanonical amino acid, *meso*-diaminopimelic acid (mDAP) in the third position[5]. The general stem peptide sequence is: L-Alanine, γ-D-Glutamic Acid, mDAP or L-Lysine, D-Alanine, D-Alanine where the letters L and D signify the stereochemistry of the amino acid.[5]

Peptidoglycan derives its mesh-like structure from crosslinks formed between stem peptides on adjacent glycan chains. The class of enzymes responsible for catalyzing this crosslinking reaction is called the penicillin binding proteins (PBPs)[6]. PBPs catalyze the formation of a bond between the third position residue of one stem peptide and the fourth position D-Alanine of another, acting on a pentapeptide substrate (the terminal D-alanine is removed by the enzyme during the reaction)[6]. Alternatively, these enzymes are known as D,D Transpeptidases because they cleave the stem peptide between the terminal two amino acids, each having a D stereocenter[6]. PBPs are named as such because they are inhibited by penicillin and other β-Lactam antibiotics[7]. These antibiotics act as suicide inhibitors, mimicking the structure of the D-Alanine, D-Alanine portion of a stem pentapeptide substrate and binding irreversibly to these essential enzymes[7]. Without the ability to properly crosslink its cell wall, the bacterial cell dies due to osmotic imbalance and environmental stress.

Resistance to β-Lactam drugs is widely documented and arises through multiple different mechanisms; however, the method relevant to this discussion is an alternate class of enzymes known as the L,D Transpeptidases (Ldts).[8] First identified in the bacteria species *E. faecium*, Ldt activity has since been discovered in other species including *E. faecalis*, *B. subtilis*, and *M. tuberculosis*[9-10]. Ldts catalyze the formation of a 3-3 crosslink between the third position residues on two stem peptides as opposed to the canonical 4-3 crosslinks like PBPs[8]. Instead of utilizing a pentapeptide substrate, the pentapeptide previously described is converted to a tetrapeptide by the removal of the terminal D-Alanine residue[8]. The enzymatic structure of Ldts is highly divergent from that of the PBPs, so β-Lactams are unable to act as suicide inhibitors[8]. Thus, the bacteria can to continue crosslinking their cell walls in the presence of antibiotics, making them resistant to this major class of drugs.

Although initially hailed as a panacea to cure all infections, people were quick to realize that the emergence of resistance to antibiotics posed a serious problem. In his Nobel Prize acceptance speech for the discovery of penicillin, Alexander Fleming warns that “there is the

danger that the ignorant man may easily underdose himself and by exposing his microbes to non-lethal quantities of the drug make them resistant"[11]. Indeed, the CDC reports that 2,000,000 million Americans are sickened and 23,000 Americans die annually because of antibiotic resistant bacteria[11-12].

Furthermore, the rate of the development of resistant strains of bacteria is accelerating[10]. For example, when erythromycin was introduced in 1953, resistant *Streptococci* did not emerge until fifteen years later; however, when ceftaroline came out in 2010, it took only one year for ceftaroline resistant *Staphylococci* to appear[12]. Additionally, while the incidence of resistance has increased, the number of new antibiotics being developed continues to decline[13-14]. One estimate projects that by 2050, antimicrobial resistance will be responsible for 10 million deaths per year, surpassing cancer as a leading cause of death worldwide[15]. Unless more is done to combat the issue, we could be entering a post-antibiotic era.

This project focused on developing fluorescent probes to characterize cell wall biosynthesis in a variety of bacterial species. Previous work has shown that small peptides or single amino acids conjugated to a fluorophore can serve as probes to study bacterial cross-linking *in vivo*[16]. As bacteria grown and synthesize and remodel their cell walls, the probes become covalently incorporated into their peptidoglycan, tagging the bacterial surface with a fluorophore, allowing for quantification via flow cytometry or visualization via confocal microscopy.

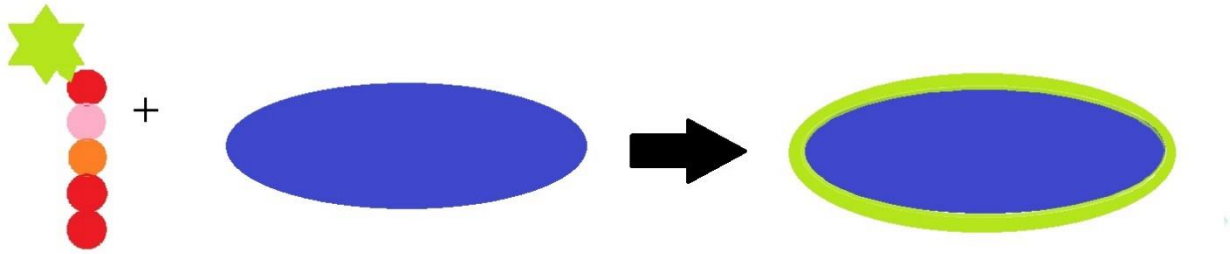


Figure 1: *Schematic depiction of bacterial cell surface labeling with fluorescent stem peptide mimics.*

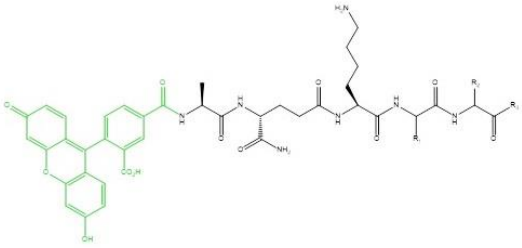
However, this study only focused on developing probes to track PBP activity in organisms containing lysine at the third position of the stem peptide[16]. Many significant human pathogens, including *M. tuberculosis* and *E. coli* contain mDAP in the third position instead of lysine[5]. Furthermore, a considerable number of resistant organisms employ Ldts during their cell wall biosynthesis[9-10]. There do not exist mDAP probe analogs nor do there exist probes to for Ldt activity. Thus, by better mimicking the natural substrates for these enzymes, we aim to further characterize transpeptidase activity in mDAP-containing organisms as well as those with a substantial degree of Ldt activity.

Ldt Probes

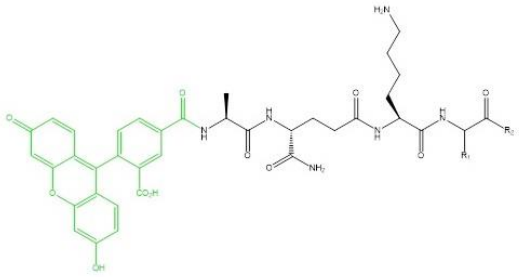
Despite the fact that Ldts play a crucial role in drug resistance mechanisms, until this point there have not been probes developed to characterize their activity *in vivo*. We aimed to remedy this by synthesizing mimics of the natural tetrapeptide substrate acted upon by these enzymes. For this library of probes, we only focused on developing probes which contained the L-lysine residue in the third position.

Methods:

The following library of probes was built using solid phase peptide synthesis.



	R_1	R_2	R_3
PentaGln-OH	D-Ala	D-Ala	OH
PentaGln-NH_2	D-Ala	D-Ala	NH_2
PentaL-OH	L-Ala	L-Ala	OH



	R_1	R_2
TetraGln-OH	D-Ala	OH
TetraGln-NH_2	D-Ala	NH_2
TetraL-OH	L-Ala	OH

Figure 2: *Library of Pentapeptides and Tetrapeptides used to characterize PBP and Ldt activity*

Probes which have a hydroxyl functionality on the C-terminus were synthesized on 2-chlorotrityl chloride resin and those which have an amino functionality on the C-terminus were synthesized on Rink Amide resin.

Synthesis/Purification:

For probes built on 2-chlorotritylchloride resin, the following protocol outlines the synthesis procedure. Amino acids were added sequentially as the peptide was built in the C to N terminal direction. For the first amino acid, 1.1 equivalents of amino acid and 4.4 equivalents of DIEA were added to 10 mL of dry DCM and placed in the peptide vessel. The vessel was agitated at room temperature for two hours. After the two hour agitation, the resin was washed with approximately 10 mL of solvents multiple times, where the pattern of solvents used was: DCM, Methanol, DCM, Methanol, DCM, DCM. Then approximately 15 mL of deprotecting solution (6 M piperazine/100 mM HOBt in DMF) was added to the peptide vessel and the vessel was agitated at ambient temperature for 25 minutes. Then the washing procedure was repeated. The second amino acid (3 equivalents), HBTU (3 equivalents), and DIEA (6 equivalents) were added to 10 mL DMF and placed in the peptide vessel which was subsequently agitated at room temperature for two hours. The washing step and deprotection step were repeated as previously described. The amino acid coupling procedure (in DMF) was repeated for each additional amino acid in the protein. After the final amino acid was added, deprotection was done as previously described using the piperazine/HOBt solution. Then, 2 equivalents of 5(6)-carboxyfluorescein, 2 equivalents of HBTU, and 4 equivalents of DIEA were added to 10 mL DMF and the solution was added to the peptide vessel. The peptide vessel was covered in aluminum foil and agitated overnight at room temperature. Washing was repeated as previously described the following morning. After washing, the resin was transferred to a 50 mL conical tube. To the conical tube was added 0.5 mL dH_2O, 0.5 mL triisopropylsilane (TIPS), and 19 mL trifluoroacetic acid (TFA). The conical tube was agitated at room temperature for two hours. Next, the solution was filtered to remove the resin and the TFA was evaporated using compressed air. Finally, the

peptide was extracted from solution using cold ether and compacted into a pellet via centrifugation.

The synthesis methods for the peptides with an amino group on the C-terminus only differed in the first steps. Rink amide resin was added to the peptide vessel to begin, and before adding any amino acids, the resin was Fmoc deprotected with the piperazine/HOBt solution. The resin was then washed, and the amino acids were coupled using the DMF-based procedure described above (3 equivalents of amino acid, 3 equivalents of HBTU, and 6 equivalents of DIEA). Addition of the 5(6)-carboxyfluorescein, cleavage from the resin, and isolation of the peptide was done using the protocol described previously.

After synthesis was completed, the peptides were purified using reverse phase high performance liquid chromatography (HPLC). Methanol and water were the solvents used to elute in the gradient. Electrospray ionization mass spectroscopy or MALDI-TOF mass spectroscopy techniques were used to identify the peptide by mass. Fractions were concentrated on the rotary evaporator, and stock concentrations were obtained by measuring absorbance at 492 nm and using the extinction coefficient for the fluorophore. Sample purity was verified by either analytical HPLC or LC-MS techniques.

Labeling Assays

Bacterial cells from an overnight culture (*M. smegmatis* cells were grown over two nights) were added in a 1:10 dilution of growth medium (Growth medium varied depending on the bacterial species being studied. *B. subtilis, E. coli,* and *S. epidermidis* were grown in LB media and *M. smegmatis* was grown in LB with 0.05% Tween80 added) containing a 100 µM concentration of the peptide probe. Conditions were set up in triplicate in a 96-well plate. The well plate was placed in a 37°C incubator shaking at 250 rpm overnight (incubation for *M. smegmatis* was done over the course of two nights). The following morning, cells were washed three times with 200 µL 1x PBS and then fixed in 200 µL of a 2% formaldehyde solution (in 1x PBS) at ambient temperature for 30 minutes. After fixation, fluorescence levels were measured using a BDFacs Canto II flow cytometer.

Results:

Species Scan for Peptide Probes

We first sought to identify the optimal organism in which to test these probes as well as to determine whether the amidation state at the C-terminus of the peptide probes had an impact on incorporation levels. The following probes were tested in this series of experiments: TetraGln-OH, TetraGln-NH_2, TetraL-OH, PentaGln-OH, PentaGln-NH_2, PentaL-OH. This library was incubated with *B. subtilis, E. coli, S. epidermidis,* and *M. smegmatis* cells using the aforementioned protocol. The fluorescence data for *B. subtilis, E. coli,* and *S. epidermidis* is depicted in Figure 3. Labeling levels were low for all conditions for *B. subtilis* cells with no departure from background signals. *E. coli* showed the greatest labeling levels for PentaGln-NH_2 while *S. epidermidis* showed the greatest labeling levels for TetraGln-NH_2 (Figure 3). However, with fluorescence levels low across the board, differences observed may be due to slight fluctuations in labeling patterns, making it difficult to reach conclusions about the nature of crosslinking in these bacteria species.

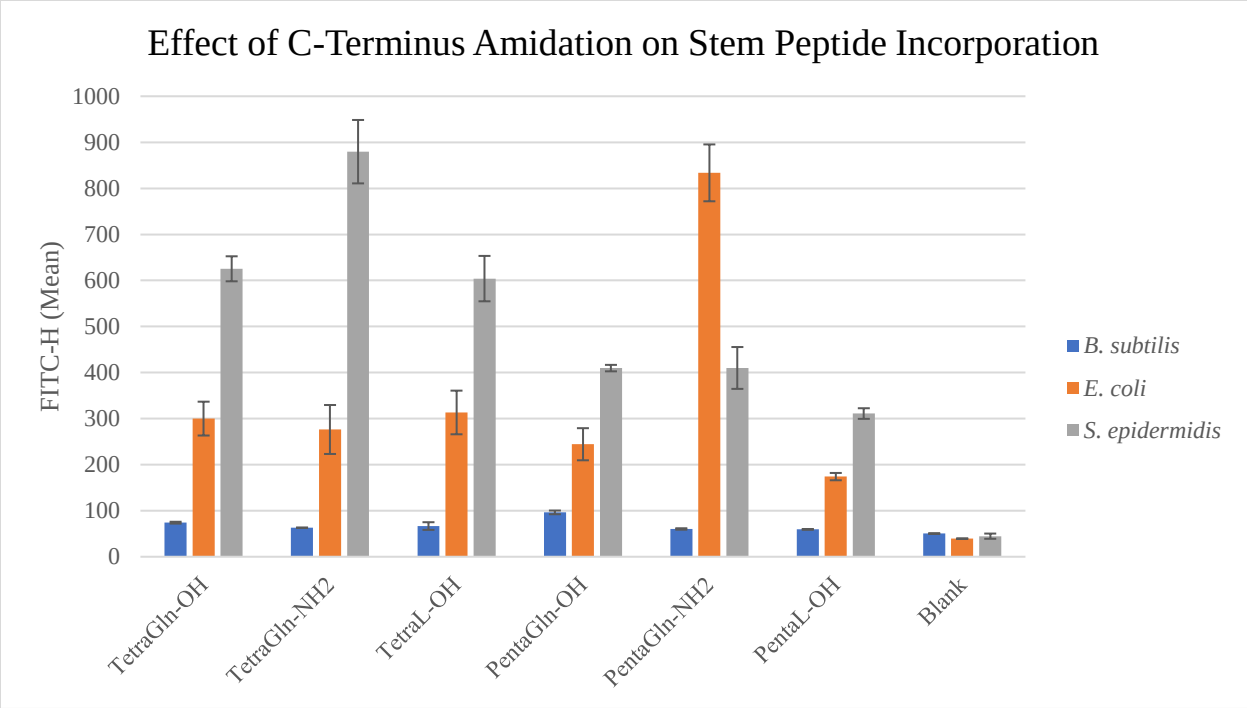


Figure 3*: This figure depicts the fluorescence levels for* B. subtilis, E. coli, *and* S. epidermidis *cells incubated with the library of tetra and pentapeptides. Labeling levels remained relatively low across all three species.*

While the results for *B. subtilis*, *E. coli*, and *S. epidermidis* yielded little in the way of conclusions about the nature of cell wall biosynthesis, the data for *M. smegmatis* proved quite surprising (Figure 4). Incorporation levels approached 30000 a.u., significantly higher than for any other species tested (Figure 4). The fact that the stem peptide mimics got incorporated into mycobacterial peptidoglycan is even more astonishing because they were able to get past the highly waxy mycolic acid layer surrounding the cell[17]. A hallmark of the *Mycobacterium* genus, these lipids protect the cell and make it nearly impermeable, one of the reasons why *Mycobacterial* infections are difficult to treat[17]. Another noteworthy trend from the *M. smegmatis* data set was the preferential incorporation of tetrapeptide over pentapeptide substrates. This could be due to different catalytic efficiencies between PBPs and Ldts, but there is precedent in the literature for extensive Ldt activity in *M. smegmatis*. *Mycobacteria* possess intrinsic drug resistance to the β-lactams due to high levels of Ldt activity[18]. In fact, Ldt-catalyzed crosslinks can comprise up to 80% of total crosslinks in *M. tuberculosis* during the bacteria's stationary phase of growth[18]. Furthermore, *M. smegmatis* showed a clear preference for peptides with a hydroxyl functionality on the C-terminus over those with the amide functionality (Figure 4). This was the expected result as the hydroxyl group is the variant present in nature.

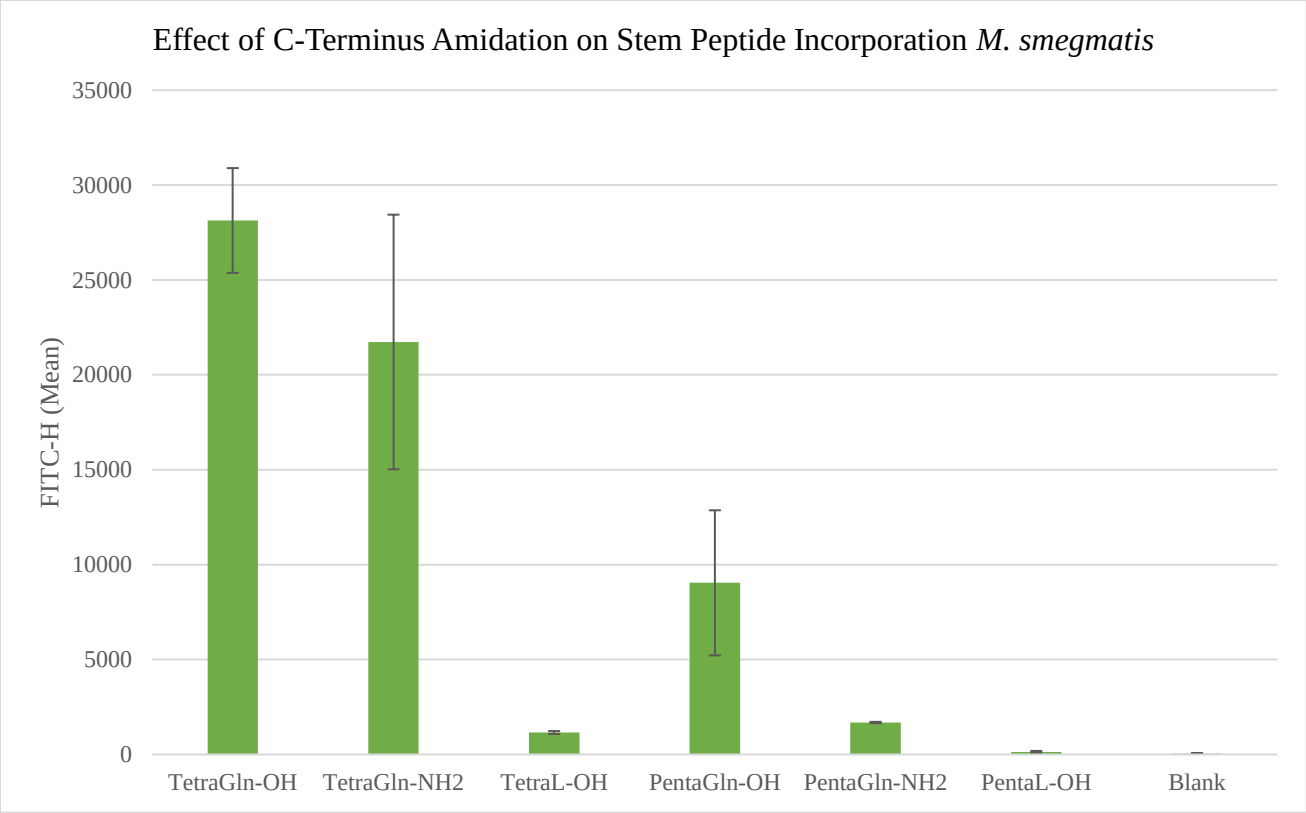


Figure 4*: This figure depicts the fluorescence levels for wild type* M. smegmatis *cells incubated over two nights with the library of tetra and pentapeptides. Significant labeling was observed, and the organisms showed a preference for the tetrapeptides over the pentapeptides.*

Thus, based on the extensive labeling levels, known Ldt activity, and clinical relevance due to its connection to *M. tuberculosis*, *M. smegmatis* was selected as the organism which would be heavily focused on for the remainder of the experiments.

M. smegmatis *Time Course Assay*

We then performed a time course assay to determine the rate at which the TetraGln-OH and PentaGln-OH probes were being incorporated by the *M. smegmatis* (Figure 5). The experiment was set up at a 100 µM probe concentration and small aliquots of cells were removed in triplicate, washed 3x with 1x PBS, and formaldehyde fixed every two hours of the day. Time points were taken after 0, 2, 4, 6, 24, 26, 28, 30, and 48 hours of incubation at 37°C (Figure 5). Over the course of the two days of the experiment, fluorescence levels rose to comparable values to those seen in the first *M. smegmatis* labeling assay with the average fluorescence reading for TetraGln-OH reaching approximately 25000 a.u. and that for PentaGln-OH reaching approximately 10000 a.u. The increase in signal was not instantaneous for either probe but occurred gradually over the course of the experiment. The signal for the PentaGln-OH appeared to level off after 24 hours, while the signal for the TetraGln-OH appeared to continue rising throughout the course of the experiment. This could potentially signal that Ldts continue to be active when the bacteria reach stationary phase, a portion of the lifecycle during which growth and division of the bacterial population slow tremendously.

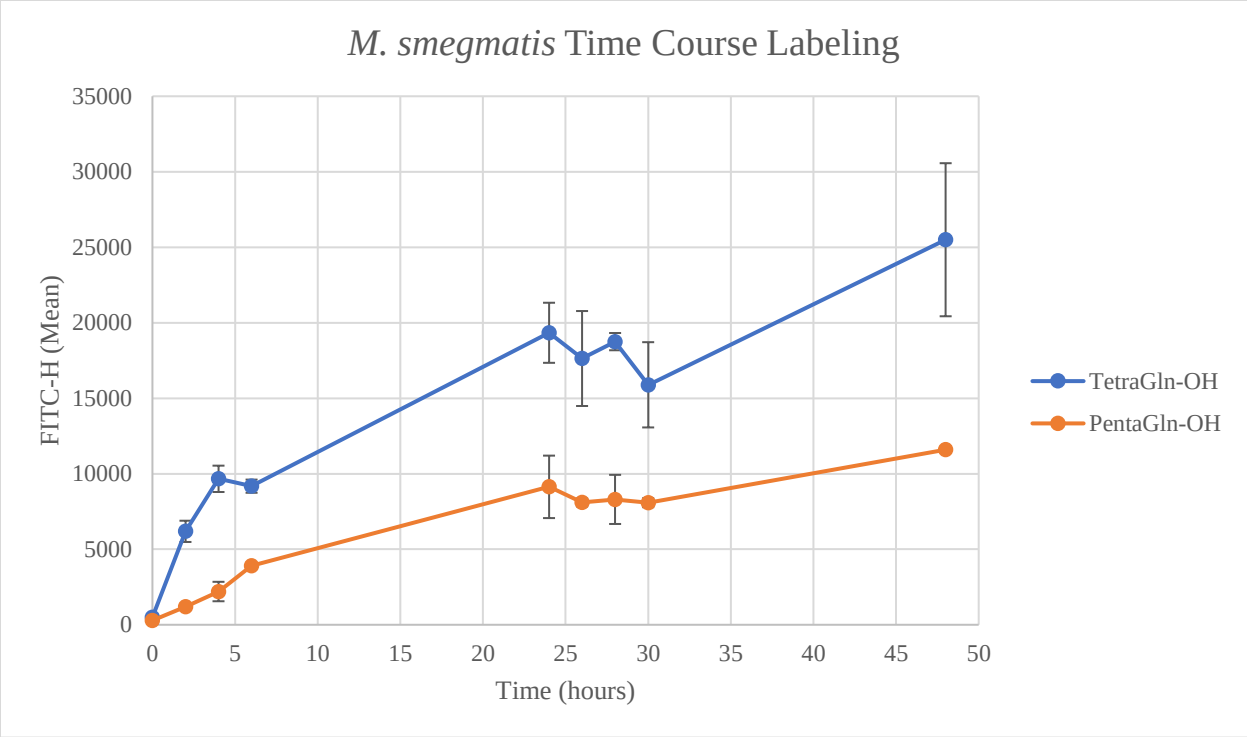


Figure 5: *Time course assay for labeling of wild type* M. smegmatis *cells with TetraGln-OH and PentaGln-OH. The assay was run over three days with time points being taken every two hours during the first two days (with overnight incubation) with a final time point taken in the morning after two nights in the incubator.*

M. smegmatis *Ldt Knockout Experiments*

As previously mentioned, Ldts are highly active in *M. tuberculosis*, a close cousin to the nonpathogenic model organism, *M. smegmatis*. *M. tuberculosis* has been shown to posses five distinct classes of Ldts while *M. smegmatis* has six types[19]. In a series of experiments aimed at further characterizing these enzymatic classes, researchers created various *M. smegmatis* knockout strains which lacked one or more of these Ldt classes[19]. To better understand the role of Ldt activity in *M. smegmatis* biosynthesis, a few of these strains were tested against our library of Ldt probes. The PM965 strain represents wild type *M. smegmatis* which has all of its Ldts intact[19]. The strain PM2110 has a single knockout of the gene for *ldtC*[19]. The PM2546 strain is lacking three Ldts: *ldtC*, *ldtB*, and *ldtF*[19]. The three Ldt knockout strains were incubated with the library of six peptides previously mentioned and the results are shown in Figure 6.

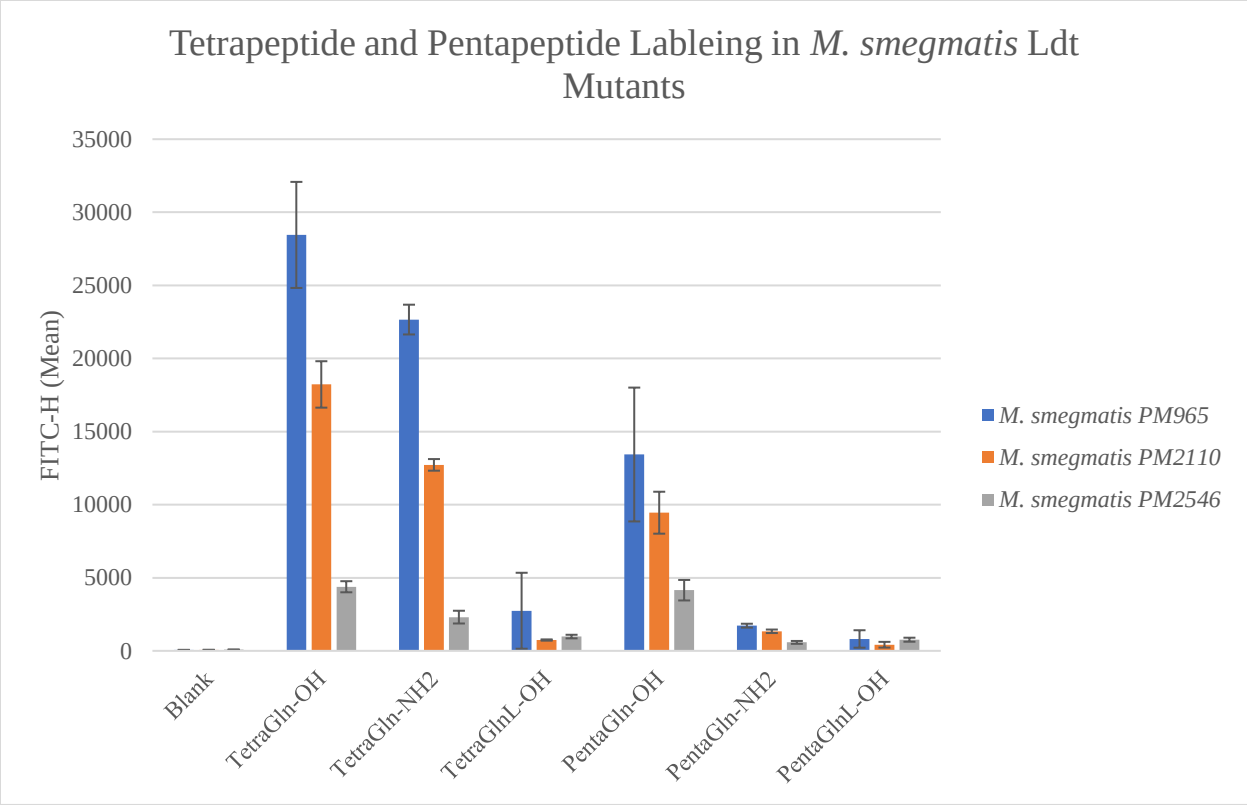


Figure 6: *Labeling of the library of pentapeptide and tetrapeptide probes in various* M. smegmatis *Ldt knockout strains. As the number of Ldt enzymes decreases, labeling with both pentapeptide and tetrapeptide probes also declines.*

As in the previous set of experiments, the *M. smegmatis* showed preferential labeling with tetrapeptides over pentapeptides. As expected TetraGln-OH had the highest incorporation levels across all strains as it is the substrate which most closely mimics the natural stem peptide of *M. smegmatis*. As the number of Ldts eliminated increased, incorporation levels decreased, again the expected result. The PM2110 strain, which lacked only one Ldt class, *ldtC*, labeled at approximately 10000 a.u. lower than the wild type strain. The result was compounded in the PM2546 strain, lacking three Ldts, where fluorescence levels were measured at approximately 4400. A similar trend was observed with TetraGln-NH_2, except labeling levels were lower in all strains. The amidation on the C-terminus of the peptide is an aberration from normal stem peptide architecture, so the lower labeling levels were expected. TetraGlnL-OH served as the negative control for the experiment as enzymes responsible for crosslinking display a high degree of stereospecificity. Changing the stereocenter from D to L on the terminal alanine residue causes a dramatic drop in incorporation levels, as evidenced by the comparison between TetraGln-OH and TetraGlnL-OH labeling levels.

The pentapeptide series of probes was aimed at measuring PBP activity in *M. smegmatis*, for pentapeptides were not known to be substrates for Ldts. Thus, it was expected that there would not be a difference in incorporation levels between strains. However, the data for the pentapeptide series displayed a similar trend to that for the tetrapeptide series. As more Ldts were knocked out when comparing between the PM965, PM2110, and PM2546 strains, incorporation levels of PentaGln-OH (and, to a lesser extent, PentaGln-NH_2) decreased as well. These data appear to suggest that perhaps Ldts can process pentapeptide substrates as well as tetrapeptides, a question which warrants future investigation.

We also wanted to test the library against only Ldt transpeptidases and eliminate residual effects of PBPs on labeling. To do so, set up a labeling assay as previously described but also included a 2mg/mL concentration of ampicillin in the growth media. Ampicillin is a member of the β-Lactam family of antibiotics and works to inhibit PBPs[20]. Since *Mycobacteria* are intrinsically resistant to these drugs, we could incubate with an exceptionally high level of ampicillin (2 mg/mL is well above the MIC value for susceptible organisms) to ensure all PBPs are inhibited. The data are shown in Figure 7. Fluorescence levels dropped for all conditions. This could be due to strain placed on the cells from growth in the exceptionally high ampicillin concentration or interexperimental variation. As was the case in the previous experiment, TetraGln-OH had the highest incorporation level, and fluorescence levels declined as more Ldts were knocked out. This further supports the idea that the tetrapeptide probes, specifically TetraGln-OH, can be used to study Ldt activity. There was not a difference in pentapeptide incorporation between the two experimental variants (PentaGln-OH and PentaGln-NH_2) and the negative control, PentaGlnL-OH (Figure 5). Thus, this signifies that PBP activity was sufficiently inhibited by the ampicillin, so the labeling observed truly corresponds to the activity of Ldts.

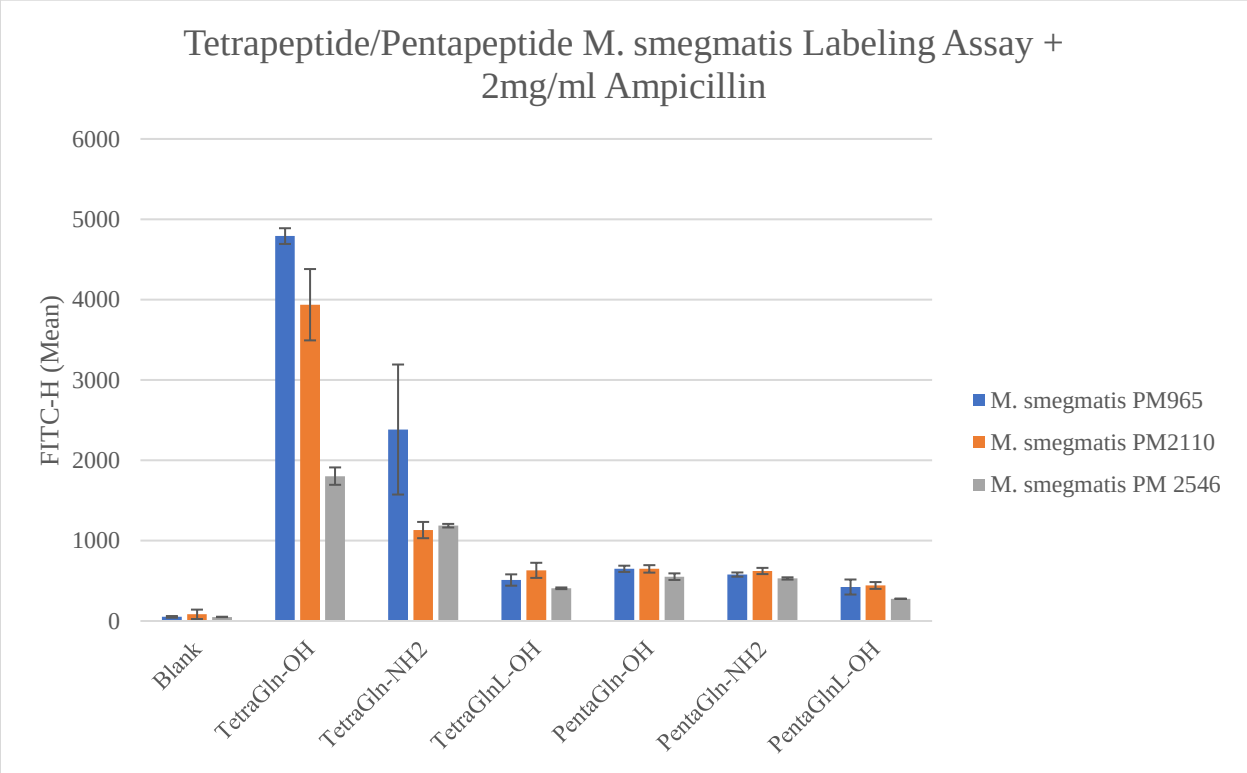


Figure 7*: Labeling of the library of pentapeptide and tetrapeptide probes in various* M. smegmatis *Ldt knockout strains incubated with 2 mg/mL ampicillin. As the number of Ldt enzymes decreases, labeling with tetrapeptide probes declines. PBPs have been inhibited in all cases by ampicillin, resulting in equally low fluorescence signals between all strains.*

Localization of PBPs and Ldts on the Bacterial Cell Wall

We next sought to simultaneously examine the activity of PBPs and Ldts. In order to do this, we needed to develop distinguishable probes for the two classes of enzymes. For the Ltd probe, we synthesized TetraGln-TMR, building the same amino acid sequence as TetraGln-OH on 2-chlorotrityl chloride resin, but instead of coupling to the fluorophore 5(6)-carboxyfluorescein, we coupled it to 5(6)-carboxytetramethylrhodamine using the same protocol

as previously described. As done previously, the peptide was purified via reverse phase HPLC. 5(6)-carboxymethylrhodamine is a pink/red fluorophore while 5(6)-carboxyfluorescein is a green fluorophore. The TetraGln-TMR was synthesized to help differentiate the signal between Ldt and PBP activity.

This experiment was run in *E. faecium* cells, another bacterial species with known Ldt activity[8]. Cells were coincubated with TetraGln-TMR and PentaGln-OH, each at a 500 μM concentration, for five minutes. The cells were then prepared for and observed under the confocal microscope. It was found that PBP activity was localized primarily to the septal region of the cell while Ldt activity was found dispersed across the entire cell surface. The coincubation experiment was also done in *M. smegmatis* with a 30 minute incubation time (as *M. smegmatis* cells grow notoriously slowly). Similar results were observed with PBP activity primarily located at the septal poles and Ldt activity spread across the surface of the cell.

The distinct localization of the two classes of enzyme indicates that perhaps they have separate roles in the cell. This is a finding which merits further investigations, for better understanding the roles of PBPs and Ldts could serve essential to designing new antibacterial agents in this era of antibiotic resistance. Credit goes to Sean Pidgeon, graduate student in the Pires Lab, for running these assays and imaging on the confocal microscope.

Discussion:

The goal of this set of experiments was to create a series of fluorescent probes which could be used to study Ldt activity *in vivo*. We synthesized a library of tetrapeptide and pentapeptide mimics of endogenous stem peptides found in bacterial peptidoglycan. The mimics included slight variations, specifically in the functionality of the C-terminus of the peptide and the stereochemistry of the terminal amino acid residues. The initial series of experiments was conducted to characterize PBP and Ldt activity in a wide variety of bacterial species. *B. subtilis*, *S. epidermidis*, *E. coli*, and *M. smegmatis* were the species studied, and *M. smegmatis* was selected as the organism to be used for future investigations due to its high labeling levels and previously documented high levels of Ldt crosslinking[18].

Besides the high levels of labeling, the initial results of *M. smegmatis* were in accord with the expected labeling patterns of the library. The cells showed a preference for incorporating tetrapeptides over pentapeptides, which, while it could be due to differing catalytic efficiencies between the enzymes, also corresponds with the abundance of 3→3 crosslinks naturally found in *Mycobacterial* cells. Furthermore, the fluorescence signal was higher for both the tetrapeptide and pentapeptide probes with a carboxylic acid functionality on the C-terminus instead of the amide functionality. Again, this was the expected result because the hydroxyl group of the carboxylic acid more closely mimics the natural substrate for these enzymes. Thus this set of results was significant in that it provided support that the exogenous mimics were being received and processed by the biosynthetic machinery in a method similar to biologically produced substrates. In other words, these results hinted that the probes developed could be used to accurately characterize bacterial cell wall biosynthesis.

The results from the *M. smegmatis* Ldt mutant strains further corroborated the idea that the tetrapeptide series of probes could be used to characterize Ldt activity. The expected result was that probe incorporation would decrease as the number of active Ldt enzymes decreased, and this was observed in the knockout versions of *M. smegmatis*. This piece of data indicates that the tetrapeptide probes are, in fact, being processed by Ldts, providing credence to the assertion that we have developed probes for Ldt activity *in vivo*.

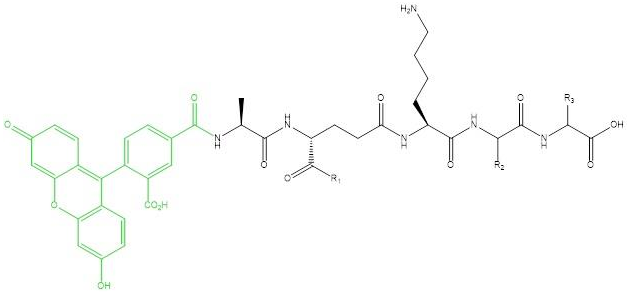
The final experiment on using Ldt probes to characterize Ldt and PBP activity *in vivo* required simultaneous visualization of the two classes of enzymes. This was accomplished by incubating the bacterial cells with a probe targeted toward PBP activity and one targeted toward Ldt activity at the same time. The PBP probe was of the series used in prior experiments, conjugated to the green fluorophore 5(6)-carboxyfluorescein while the Ldt probe had the same peptide sequence as the TetraGln-OH probe used in prior experiments, but was conjugated to 5(6)-carboxymethylrhodamine, a red fluorophore. Confocal microscopy was used to visualize both *E. faecium and M. smegmatis* cells. Both species showed distinct organization of PBPs and Ldts, with PBPs predominately located in the septal regions and Ldts active throughout the cell surface. These results were significant because it was the first evidence that these two classes of enzymes are spatially separated *in vivo*, possibly suggesting that the enzymatic classes play distinct roles in normal cellular functioning. Further investigation into the nature of PBP and Ldt localization is warranted, and the aforementioned probe library provides a potential tool for studying these activities.

Amidation State of Glutamic Acid Residue

A common modification observed in bacterial peptidoglycan is the amidation of the second position glutamic acid residue in the stem peptide[5]. It is not well known why certain bacteria enzymatically alter this residue by amidating the alpha carboxylic acid functionality on the glutamic acid residue, but perhaps it could serve as a mechanism to regulate crosslinking. The effect of amidation state on the second position amino acid was tested in *M. smegmatis*. It should be noted that the natural peptidoglycan of *Mycobacteria* is amidated at this position, hence why the Ldt probes were synthesized with an amino group on the site of interest[5].

Methods:

The following library of three pentapeptide probes (note, some were also used for the Ldt experiments) was built using solid phase peptide synthesis.



	R_1	R_2	R_3
PentaGln-OH	NH_2	D-Ala	D-Ala
PentaGlu-OH	OH	D-Ala	D-Ala
PentaL-OH	NH_2	L-Ala	L-Ala

***Figure 8**: Pentapeptide probes used to determine the effect of amidation on the second residue on crosslinking of peptidoglycan*

All probes for this section were synthesized on 2-chlorotrityl chloride resin.

Synthesis/Purification:

The following protocol outlines the synthesis procedure. Amino acids were added sequentially as the peptide was built in the C to N terminal direction. For the first amino acid, 1.1 equivalents of amino acid and 4.4 equivalents of DIEA were added to 10 mL of dry DCM and placed in the peptide vessel. The vessel was agitated at room temperature for two hours. After the two hour agitation, the resin was washed with approximately 10 ml of solvents multiple times, where the pattern of solvents used was: DCM, Methanol, DCM, Methanol, DCM, DCM. Then approximately 15 mL of deprotecting solution (6 M piperazine/100 mM HOBt in DMF) was added to the peptide vessel and the vessel was agitated at ambient temperature for 25

minutes. Then the washing procedure was repeated. The second amino acid (3 equivalents), HBTU (3 equivalents), and DIEA (6 equivalents) were added to 10 mL DMF and placed in the peptide vessel which was subsequently agitated at room temperature for two hours. The washing step and deprotection step were repeated as previously described. The amino acid coupling procedure (in DMF) was repeated for each additional amino acid in the protein. After the final amino acid was added, deprotection was done as previously described using the piperazine/HOBt solution. Then, 2 equivalents of 5(6)-carboxyfluorescein, 2 equivalents of HBTU, and 4 equivalents of DIEA were added to 10 mL DMF and the solution was added to the peptide vessel. The peptide vessel was covered in aluminum foil and agitated overnight at room temperature. Washing was repeated as previously described the following morning. After washing, the resin was transferred to a 50 mL conical tube. To the conical tube was added 0.5 mL dH_2O, 0.5 mL triisopropylsilane (TIPS), and 19 mL trifluoroacetic acid (TFA). The conical tube was agitated at room temperature for two hours. Next, the solution was filtered to remove the resin and the TFA was evaporated using compressed air. Finally, the peptide was extracted from solution using cold ether and compacted into a pellet via centrifugation.

After synthesis was completed, the peptides were purified using reverse phase high performance liquid chromatography (HPLC). Methanol and water were the solvents used to elute in the gradient. Electrospray ionization mass spectroscopy or MALDI-TOF mass spectroscopy techniques were used to identify the peptide by mass. Fractions were concentrated on the rotary evaporator, and stock concentrations were obtained by measuring absorbance at 492 nm and using the extinction coefficient for the fluorophore. Sample purity was verified by either analytical HPLC or LC-MS techniques.

Labeling Assays

M. smegmatis cells from a culture grown over two nights were added to 200 µL LB with 0.05% Tween80 containing a 100 µM concentration of the peptide probe. Conditions were set up in triplicate in a 96-well plate. The well plate was placed in a 37°C incubator shaking at 250 rpm and left in the incubator over two nights. After the incubation period, cells were washed three times with 200 µL of 1x PBS and then fixed in 200 µL of a 2% formaldehyde solution (in 1x PBS) at ambient temperature for 30 minutes. After fixation, fluorescence levels were measured using a BDFacs Canto II flow cytometer.

Results:

The purpose of this experiment was to determine the effect of the amidation state of the glutamic acid residue on the second position of the stem peptide on crosslinking. In this investigation, we focused on PBP activity, using pentapeptide probes instead of tetrapeptides. Thus, labeling was only tested in wild type *M. smegmatis*. The results are depicted in Figure 9. As observed in the Ldt experiments, PentaGln-OH labeled significantly in the *M. smegmatis* despite the cells' waxy outer covering. Strikingly, however, the signal from the PentaGlu-OH condition dropped back to the baseline level. In fact, there was not a significant difference in signal from the PentaGlu-OH condition and the blank cells which weren't exposed to any fluorescent probe (Figure 9). These results clearly indicate that amidation of the glutamic acid residue is essential for crosslinking by PBPs in *M. smegmatis.*

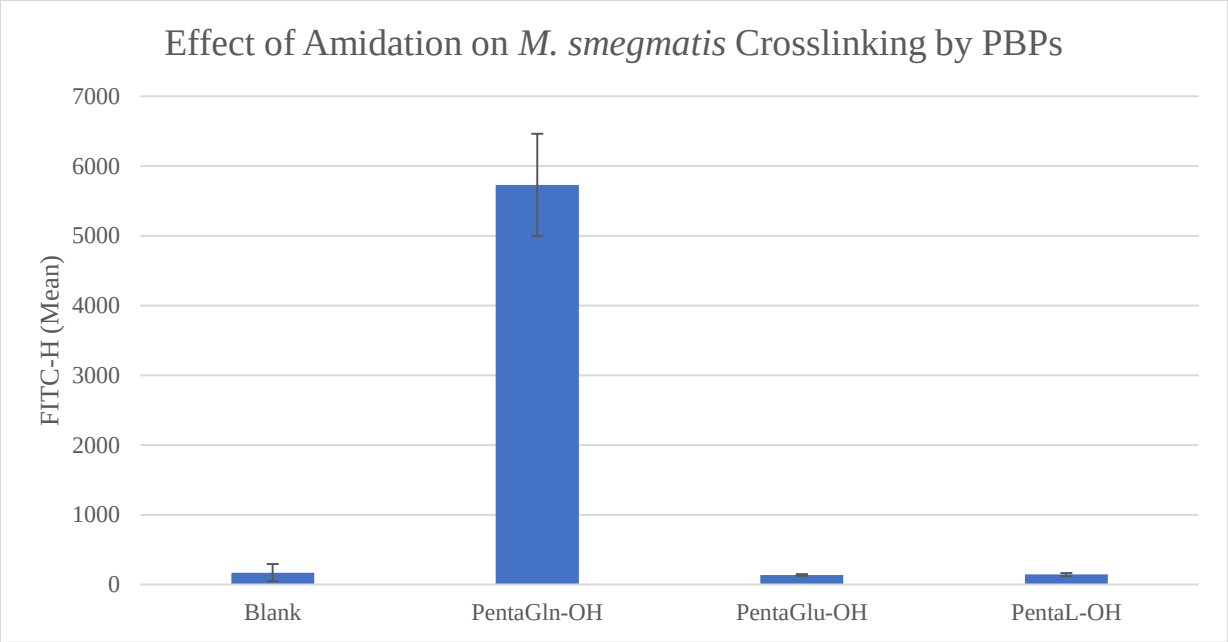


Figure 9: *Effect of amidation on the second position glutamic acid residue on crosslinking by PBPs in* M. smegmatis.

These results were also verified via confocal microscopy, and a fluorescent signal was only observed in *M. smegmatis* cells incubated with a peptide amidated at the position of interest.

Discussion:

While simple in experimental design, this set of data indicates that the amidation state of the glutamic acid residue in the stem peptide plays a major role in controlling crosslinking in *M. smegmatis* cells. Crosslinking by PBPs appears to completely cease when the residue is not amidated. As previously mentioned, since crosslinking is essential for the maintenance of cell wall integrity and bacterial cell survival, these results point towards another possible target for antibiotics. In fact, it has previously been shown that the amidation state of the glutamic acid residue is crucial for determining whether crosslinking occurs in another human pathogen, *S. pneumoniae*[22]. Deletion of the enzymes responsible for amidating the glutamic acid residue, MurT/GatD, proved lethal in *S. pneumoniae*[23]. Shortly after this assay on the importance of amidation state was run, a paper was published which described how amidation impacted crosslinking in both *E. faecium* and *M. tuberculosis*[24]. The paper details that without the proper amidation state on the glutamic acid residue or the third position side chain, crosslinking ceases completely in the organisms[24]. Thus, the enzymes responsible for amidating these positions appears a salient target for antibiotic compounds since crosslinking is such an essential function.

mDAP Mimics

Since probes to mimic lysine-containing organisms can be easily synthesized via solid phase peptide synthesis, the question arises as to why similar molecules have not been developed for those organisms which contain mDAP at the third position. mDAP is an noncanonical amino acid with two stereocenters and an internal plane of symmetry. The molecule is not commercially available, and it can only be made through a multistep synthesis; thus, this is the largest hurdle for creating mDAP containing probes via standard peptide synthesis methods[25]. However, it has recently been shown that *E. coli* will incorporate stem peptide mimics containing

meso-lanthionine at the third position into their peptidoglycan, indicating there is some flexibility in the substrates the cross-linking machinery will accept[26]. Thus, we sought to synthesize and test probe to mimic the stem peptide of mDAP containing organisms by using a disulfide functionality at the third position to create a structural analog to mDAP which can be easily synthesized using commercially available building blocks.

The structure of mDAP is depicted in Figure 10. Observe that the molecule is symmetrical and contains a similar structure to standard amino acids. In fact, the molecule looks like two amino acids bonded together.

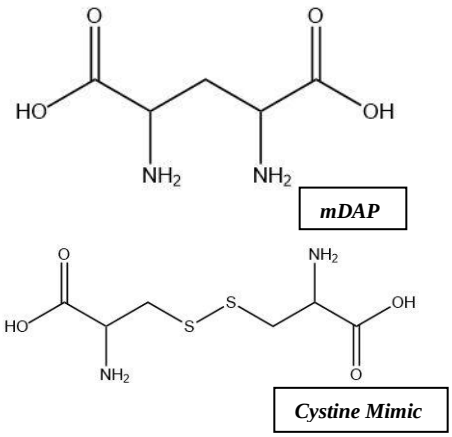


***Figure 10**: Structure of mDAP (above) and disulfide-containing mimic (below).*

The mimic we designed is shown in the adjacent figure and is labeled as Cystine Mimic. Observe that the ends of the molecule are identical to the ends of mDAP. The difference is that the cystine mimic is longer than mDAP by two atoms, and also that the cystine mimic contains a disulfide functionality as a bridge. As previously mentioned, the bacterial crosslinking machinery displays some flexibility, allowing for incorporation of sulfur containing mimics[26]. Indeed, some bacterial species contain meso-Lanthionine, a sulfur containing analog similar to our probe but one atom shorter (the bridge between the two ends consists of a single sulfur molecule instead of a disulfide bond) naturally in their third position[5]. Due to this flexibility, we anticipated that our design should be able to be processed by the crosslinking enzymes and incorporated into the cell wall.

Methods:

Synthesis/Purification:

The following series of pentapeptide probes (shown in the panel below) was synthesized for this series of experiments. mDAP is a molecule with two stereocenters (although, overall, it is achiral due to an internal mirror plane), so the probe Penta LD Cystine is the variant that most accurately matches the stereochemistry of the natural peptidoglycan. Penta LL Cystine is a probe variant which reverses the stereochemistry on the side chain of the third position residue and will be used to test the stereospecificity of the bacterial crosslinking enzymes. Amidation of the alpha carboxyl group is a chemical modification to the stem peptide naturally observed in some bacterial species, so the probe Penta LD Cystine Amide is designed to test whether the amidation state of the side chain has any impact on crosslinking[5].

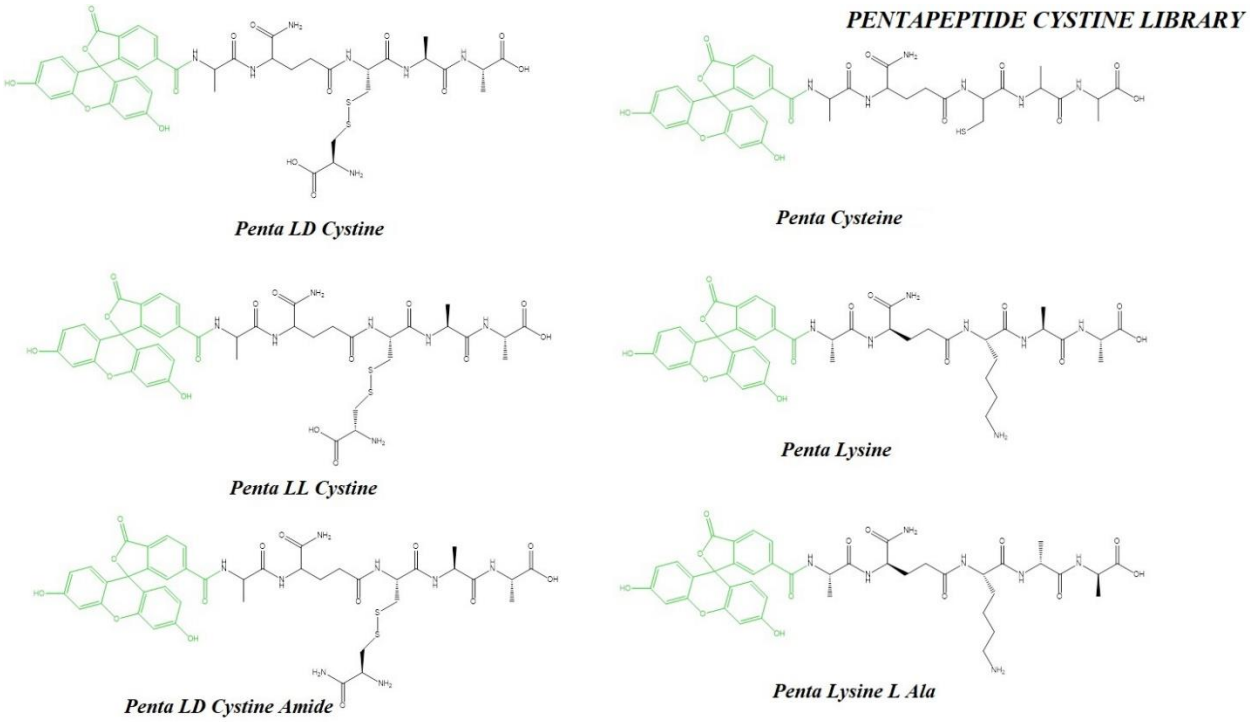


Figure 11: *Library of pentapeptides used to characterize the importance of the third position amino acid in bacterial cell crosslinking.*

Additionally, the following library of tetrapeptides was synthesized. Certain mDAP containing organisms such as *M. smegmatis* also contain Ldts, so incorporation levels of the mimics could be preferentially measured using these variants.

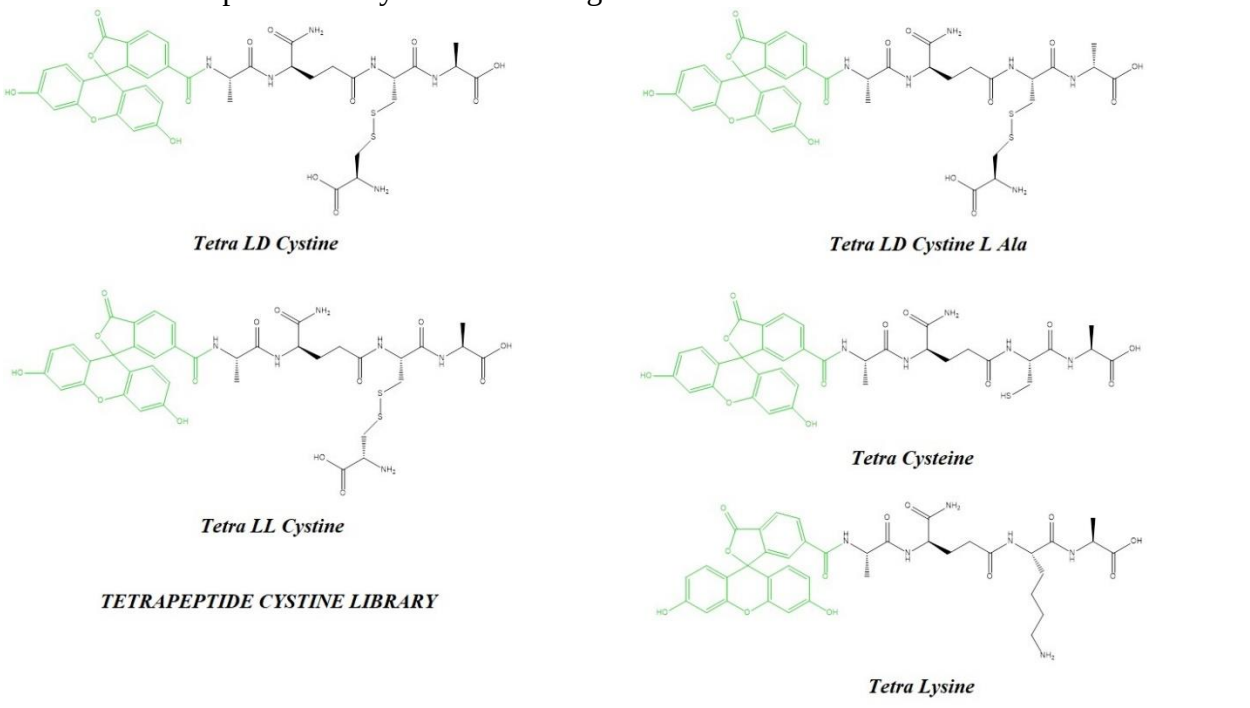


Figure 12: *Library of tetrapeptides used to characterize the importance of the third position amino acid in bacterial cell crosslinking.*

The probes Penta Cysteine, Penta Lysine, Penta Lysine L Ala, Tetra Cysteine, and Tetra Lysine were synthesized using the previously described solid phase peptide synthesis protocol. All probes were synthesized on 2-chlorotrityl chloride resin. All peptides were conjugated to the fluorophore 5(6)-carboxyfluorescein and cleaved off the resin using a 95% TFA solution. Peptides were purified on the reverse phase HPLC.

The synthesis for the probes Penta LD Cystine, Penta LL Cystine, Tetra LD Cystine, and Tetra LL Cystine was slightly more involved. The backbone for all peptides—5(6)-carboxyfluorescein, L-alanine, D-glutamic acid α amide, L-cysteine, D-alanine, D-alanine—was built on 2-chlorotrityl chloride resin. After the overnight coupling of the fluorophore, the peptides were cleaved in TFA as previously described.

For Penta LD Cystine and Tetra LD Cystine (Penta LL Cystine and Tetra LL Cystine), the peptide pellet was dissolved in 0.1 M ammonium bicarbonate solution. 5 equivalents of D-cystine (L-cystine) was dissolved in 0.1 M NaOH. Equal volumes of ammonium bicarbonate and sodium hydroxide were used to dissolve the peptide and the cystine depending on the scale of the peptide being produce (the average volume was approximately 20 mL of each). The ammonium bicarbonate solution and cystine solution were reacted in a round bottom flask with stirring at room temperature for two hours. Alkaline reaction conditions were used to deprotonate the sulfur atom on the cysteine side chain of the peptide in order to increase its nucleophilicity. A nucleophilic attack is performed by the negatively charged sulfur group on one of the two sulfur atoms in the cystine molecule, releasing free cysteine and forming the disulfide containing mimic. This process is depicted schematically below (Figure 13).

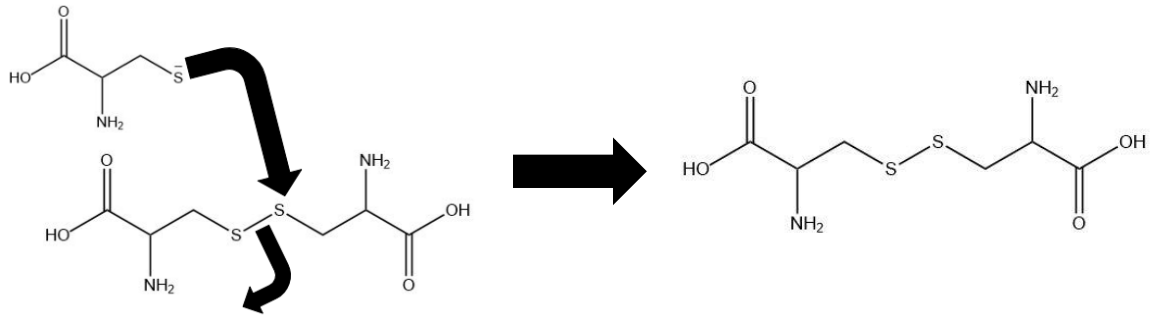


Figure 13: *Schematic diagram of nucleophilic substitution reaction used to create disulfide containing mDAP mimic.*

After two hours, solvents were evaporated using the rotary evaporator. Samples were acidified with a small volume of HCl and then purified on the HPLC using water and methanol. The gradient used for purification in this case remained at 100% water for 15 minutes before changing solvent composition to elute all salts present in solution.

For the probe Penta LD Cystine Amide, the peptide backbone was synthesized on 2-chlorotrityl chloride resin and cleaved using 95% TFA solution after coupling to the fluorophore. 5 equivalents of H-D-Cys-(OMe) were dissolved in ammonia/methanol in a small round bottom flask. The mixture was stirred at room temperature for approximately one week. Every few days the old solvent was removed with the rotary evaporator and fresh solvent was added. After a week, mass spectroscopy indicated complete hydrolysis of the methyl ester groups to amino groups by the ammonia, so this amidated cystine was dissolved in 0.1 M NaOH, the peptide dissolved in 0.1 M ammonium bicarbonate, and the synthesis/purification protocol for the other cystine-containing peptide probes was followed.

Labeling Assays

Bacterial cells from an overnight culture (*M. smegmatis* cells were grown over two nights) were added to 200 µL growth medium (Growth medium varied depending on the bacterial species being studied. *B. subtilis* was grown in LB media, *L. plantarum* and *L. casei* were grown in MRS broth, and *M. smegmatis* was grown in LB with 0.05% Tween80 added) containing a 100 µM concentration of the peptide probe. Conditions were set up in triplicate in a 96-well plate. The well plate was placed in a 37°C incubator shaking at 250 rpm overnight (incubation for *M. smegmatis* was done over the course of two nights). The following morning, cells were washed three times with 200 µL 1x PBS and then fixed in 200 µL of a 2% formaldehyde solution (in 1x PBS) at ambient temperature for 30 minutes. After fixation, fluorescence levels were measured using a BDFacs Canto II flow cytometer. Heat killed cells were boiled in a hot water bath for approximately 15 minutes before being inoculated into the well plates.

Results:

Pentapeptide Cystine Labeling of M. smegmatis

The purpose of this experiment was to determine whether organisms which contain mDAP at the third position would show a preference for a closer mimic to the natural stem peptide structure than the lysine containing probes previously tested. *M. smegmatis*, like all *Mycobacteria*, has mDAP in the third position of its stem peptide[5]. *M. smegmatis* cells were incubated over two nights with Penta LD Cystine, Penta Lysine, Penta Lysine L-Ala (negative control), and Penta Cysteine. The results from this assay are depicted in Figure 14. Labeling levels for Penta Lysine L-Ala were low, which was the expected result because of the altered stereochemistry on the terminal two residues. Additionally, labeling levels for Penta Cysteine were low, indicating that the length of the residue in the third position is important for incorporation. Thus, the extra carboxylic acid or amide group present on the end of the mDAP residue plays an important function in cell wall synthesis. The salient point of comparison was between the probes Penta Lysine and Penta LD Cystine. As shown in the Ldt series of experiments, *M. smegmatis* cells will incorporate lysine-containing probes into their cell walls. However, this experiment showed that the *M. smegmatis* cells preferentially incorporated the Penta LD Cystine to the Penta Lysine, indicating that the bacteria prefer a closer mimic to their natural stem peptide structure.

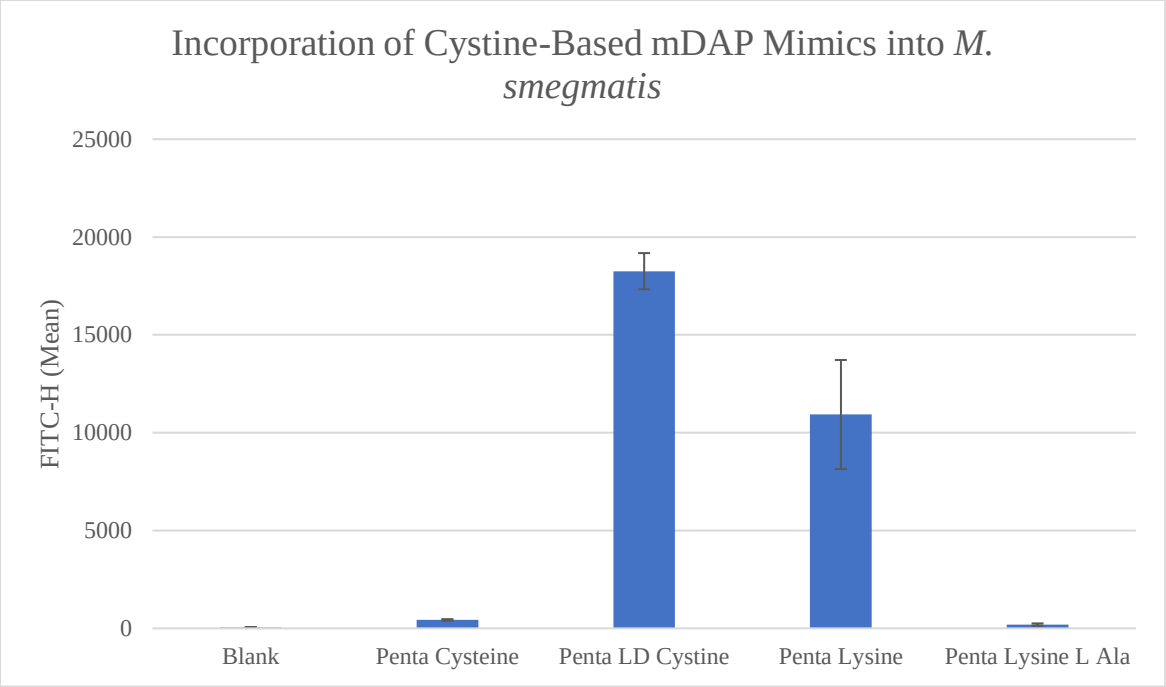


Figure 14: *Labeling of pentapeptide library in* M. smegmatis.

Pentapeptide Cystine Labeling in Multiple Species

The probes Penta Cysteine and Penta LD Cystine were tested in multiple other species to see whether the results observed in *M. smegmatis* generalize to other types of bacteria. *B. subtilis, L. plantarum,* and *L. casei* are all organisms which naturally contain mDAP in their stem peptides[5]. The results from this labeling assay are depicted in Figure 15. Based on the *M. smegmatis* data, Penta Cysteine was selected as a negative control, meaning that low labeling levels were expected with this peptide because it lacked the full side chain. In line with the hypothesis, labeling levels were statistically significantly lower for cells incubated with Penta Cysteine than with Penta LD Cystine. Thus, these results serve as further evidence that the molecule Penta LD Cystine could be used to label mDAP containing organisms. An important point to note is that Penta LD Cystine achieved significant fluorescence levels in *B. subtilis* during this assay. For reasons unknown, this species of bacteria is not well labeled with most other stem peptide mimics, so the ability of Penta LD Cystine to become incorporated in to the cell wall of *B. subtilis* is a significant finding.

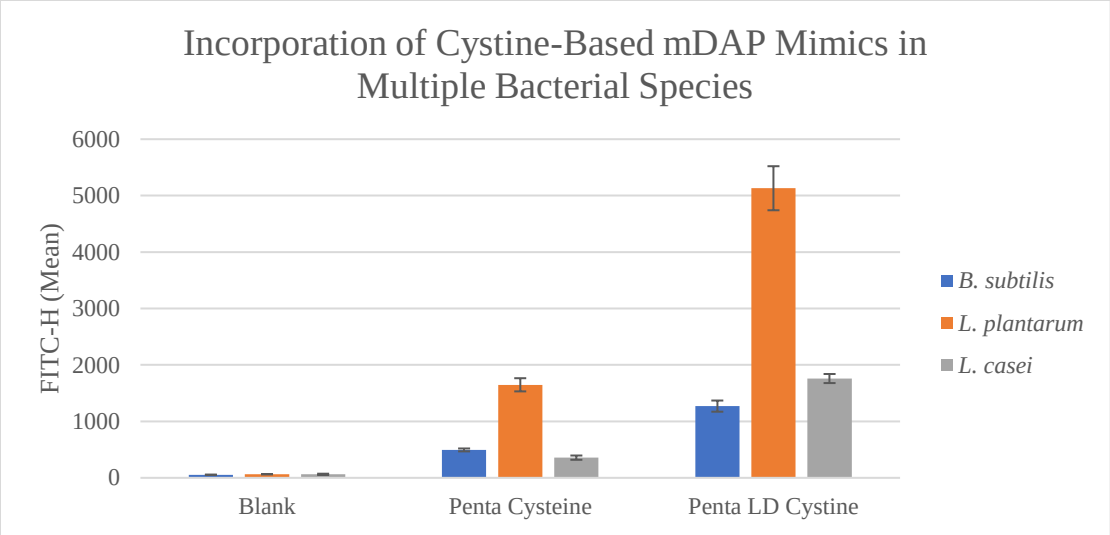


***Figure 15**: Labeling of pentapeptide library in the mDAP containing organisms* B. subtilis, L. plantarum, *and* L. casei

Variation of mDAP Side Chain

Due to the high labeling levels and clinical significance (as *M. smegmatis* is a close relative to the organism which causes tuberculosis), the next significant experiment was performed in *M. smegmatis*. This assay attempted to determine the effect of small modifications to the side chain of the third position residue on labeling levels. The data are graphically depicted in Figure 16. Penta LD Cystine, the closest mimic of the endogenous peptide of *M. smegmatis* displayed the highest labeling levels, with a high signal of 100,000 a.u. Penta LL Cystine and Penta LD Cystine Amide each labeled at approximately half the level of the perfect mimic, indicating that the biosynthetic machinery is somewhat flexible with regard to the third position side chain. This is not completely unexpected as Penta Lysine was previously shown to label in the Ldt series of experiments. Labeling levels for Penta Lysine were slightly lower in this assay then they have been in previous experiments, and Penta Cysteine and Penta Lysine L-Ala served as negative controls indicating background fluorescence levels. An interesting piece of data from this assay was the rather significant signal given by heat killed cells. Despite boiling the cells for 15 minutes, labeling levels still exceeded 30,000 a.u.

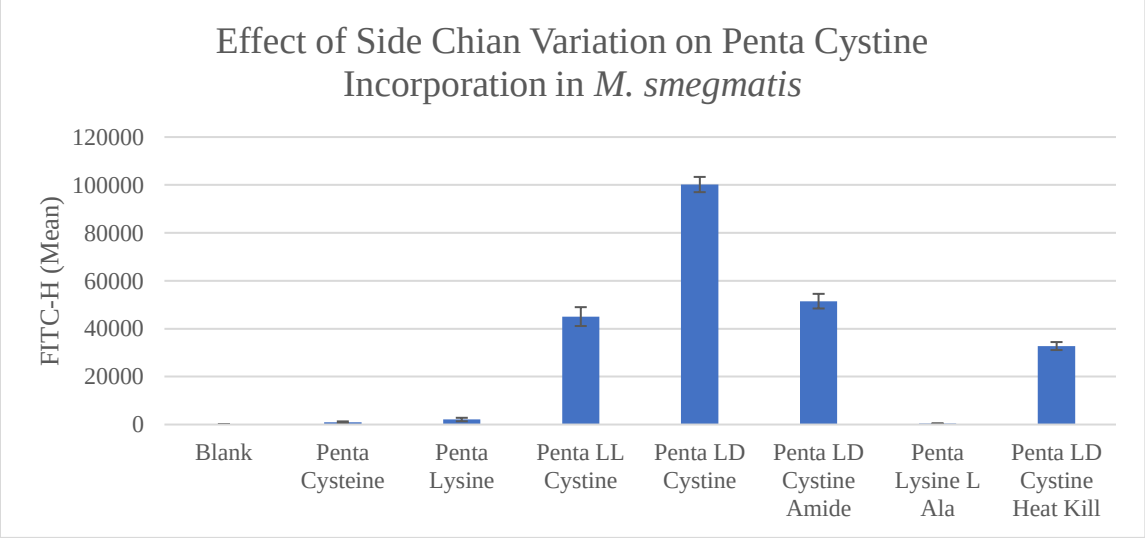


***Figure 16:** Effect of third position side chain on PBP-mediated crosslinking in* M. smegmatis

The unusual data point from the heat killed cells provided impetus to re-examine experimental setup and design. *M. smegmatis*, while clinically relevant due to its close relationship to the pathogen *M. tuberculosis*, grows very slowly making it cumbersome to run a series of labeling experiments. Another organism of interest, identified in the preliminary screen, was *L. casei*. *L. casei* is a gram positive organism which has a peptidoglycan stem peptide that also contains mDAP in the third position[5]. Furthermore, an NCBI Protein BLAST search revealed that *L. casei* contains Ldts[27]. These organisms can be grown more quickly than *M. smegmatis* and yield highly homogenous populations, helping mitigate the variation observed in the flow cytometer.

L. casei *Labeling with Cystine Library*

This experiment was run to determine whether *L. casei*, an mDAP-containing organism, showed a preference for the cystine mDAP mimic over the fluorescent probes containing lysine. Furthermore, we wanted to investigate the activity of Ldts and PBPs in these cells by testing labeling levels with both pentapeptides and tetrapeptides (Figure 17).

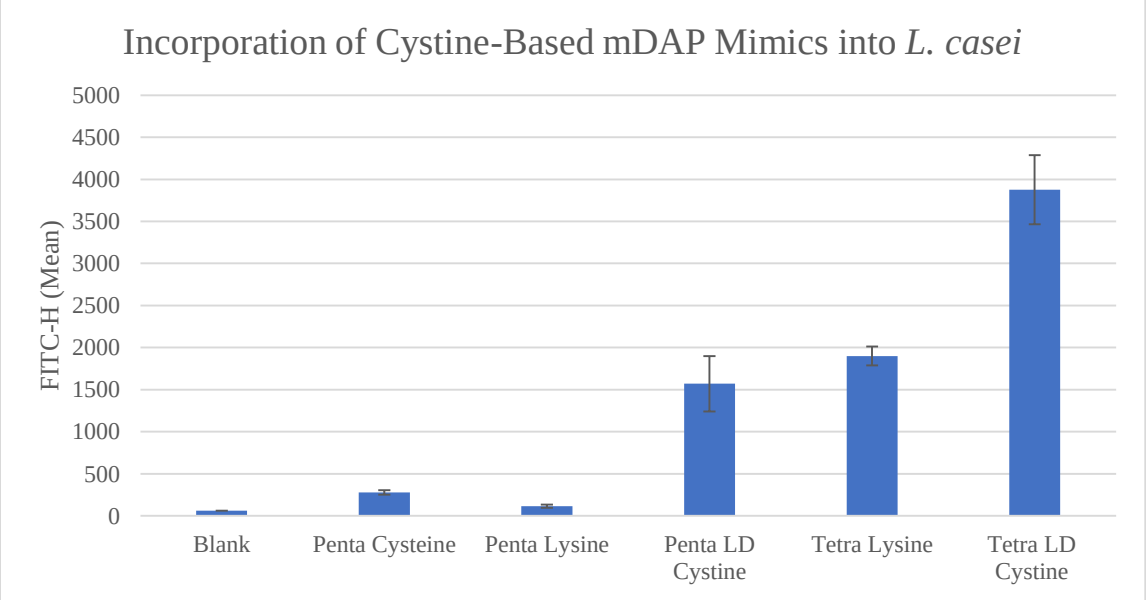


***Figure 17**: Labeling of* L. casei *cells with a library of cystine and lysine containing pentapeptides and tetrapeptides*

In the series of pentapeptides tested, *L. casei* showed a strong preference for the mDAP mimic probe to the lysine-containing probe, which barely labeled above basal fluorescence levels (Figure 17). This indicates that the PBPs responsible for peptidoglycan in *L. casei* show a high degree of specificity for the natural substrate and are not as flexible in the substrates they will accept as those of *M. smegmatis*. Surprisingly, labeling of both tetrapeptides in *L. casei* was significantly higher than that for the pentapeptides, indicating considerable Ldt activity. The cystine mimic tetrapeptide was incorporated at a higher rate than the lysine containing tetrapeptide, signifying that the Ldts in *L. casei* also preferred a closer mimic to the natural substrate. This experiment verified that *L. casei* would be a good model organism in which to test these probes.

The next assay sought to investigate the effect of slight variations on the third position residue side chain on crosslinking efficiency by PBPs and Ldts. *L. casei* cells from an overnight culture were incubated with the full library of probes: Penta Cysteine, Penta LD Cystine, Tetra Cysteine, Tetra LL Cystine, Tetra LD Cystine, and Tetra LD Cystine L Ala. Heat killed *L. casei*

cells were also incubated with the probes closest to the natural substrate, Penta LD Cystine and Tetra LD Cystine. Due to the higher labeling levels of the tetrapeptide variants identified in the previous experiment, tetrapeptides were selected as the predominant probes of interest for this experiment. Results of this assay are depicted below in Figure 18.

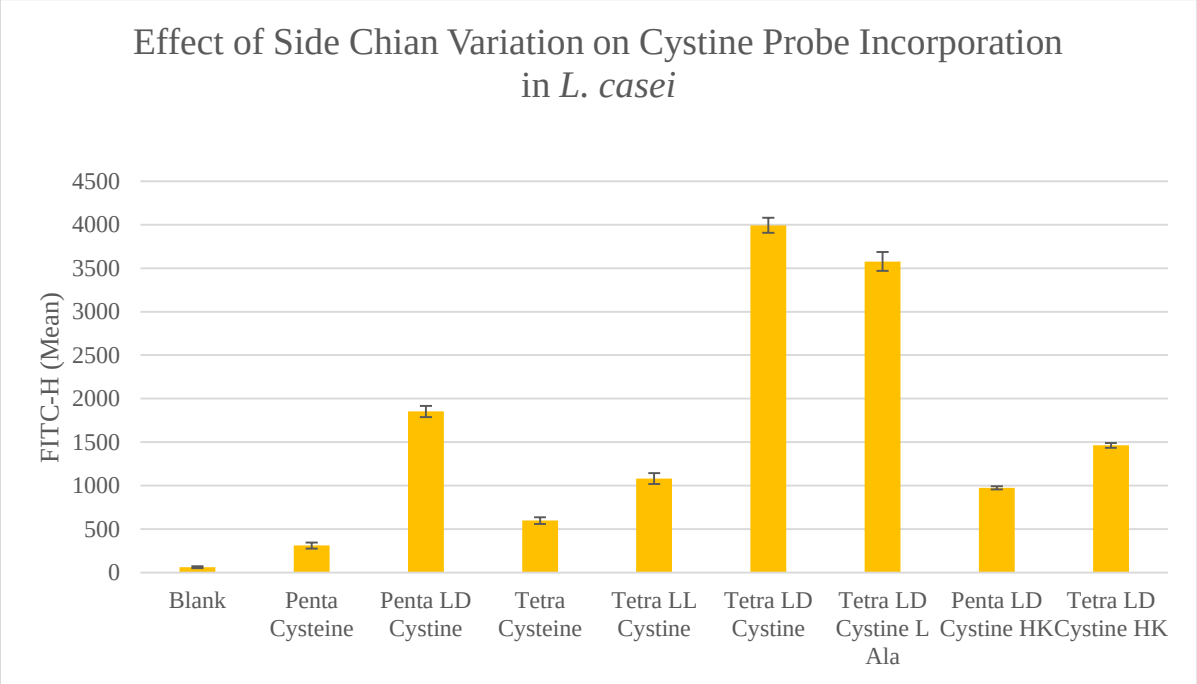


Figure 18: L. casei *cells labeled with a variety of pentapeptide and tetrapeptide probes with variations on the side chain of the third position residue.*

The pentapeptides tested recapitulated the results from the previous assay in that the Penta LD Cystine was incorporated at much higher levels than the Penta Cysteine probe. A similar trend was observed when comparing the Tetra LD Cystine and Tetra Cysteine levels. This indicates that the terminal portion of the third position residue which has similar structure to an amino acid is important for crosslinking by both PBPs and Ldts. The series of tetrapeptides tested examined the importance of the stereochemistry of the terminal portion of the third position residue. The naturally found stereochemistry of mDAP is LD, with the D stereocenter being located further from the peptide backbone. As expected, the Tetra LD Cystine probe labeled at a higher level than the Tetra LL Cystine probe. An unexpected result was the high labeling by the Tetra LD Cystine L Ala probe. In all past assays, switching the stereocenter of the terminal amino acid caused labeling to diminish dramatically and has served as a strong negative control, so the incorporation of this probe by *L. casei* was surprising and warrants further investigation. Additionally, as observed in *M. smegmatis*, heat killed *L. casei* cells still labeled significantly with both Penta LD Cystine and Tetra LD Cystine. Thus, this unusual finding generalizes to multiple species of bacteria and will be detailed further in the discussion section.

Discussion:

The purpose of this series of experiments was to design a fluorescently-conjugated stem peptide probe which mimicked the stem peptides in organisms that contain mDAP. The fact that mDAP is not commercially available and is arduous to synthesize has inhibited the development of probes specifically designed to characterize biosynthetic activity in the cell walls of mDAP

containing organisms. However, we have exploited the nucleophilicity of the thiol group on the cysteine side chain and the flexibility of the peptidoglycan crosslinking machinery to create a disulfide-containing probe which can be incorporated into the peptidoglycan of mDAP-containing organisms during their growth phase. This probe design is significant because the stem peptide backbone can be easily synthesized on a solid-support resin and adding the extra side chain piece to the third position residue can be accomplished by a simple nucleophilic substitution reaction. Furthermore, all components required to build this molecule can be purchased commercially, making these a practical method for studying mDAP-containing organisms.

Since *M. smegmatis* served as a useful model organism for characterizing Ldt activity and is known to have mDAP in its peptidoglycan, the first question we attempted to answer was whether the *M. smegmatis* crosslinking machinery would accept, and preferentially so, our cystine mimic. Along with some negative controls, we incubated *M. smegmatis* cells with two pentapeptide probes of interest: one containing lysine in the third position and the other containing the cystine mDAP mimic. While both probes were incorporated into the peptidoglycan, the fluorescence reading was higher for the mDAP mimic than for the lysine variant. This confirms the hypothesis that the PBP crosslinking machinery would prefer an exogenous substrate with a higher degree of structural similarity to the natural substrate. This result indicated that the cystine derivative we designed and synthesized could feasibly be used to mimic mDAP in live cells. We tested the probe in other mDAP containing organisms (*B. subtilis, L. plantarum,* and *L. casei*) and found that their crosslinking enzymes all accepted it as a substrate.

This initial set of experiments indicated that there was some flexibility in the molecules that the PBPs and Ldts of these organisms would accept as substrates. To further explore this property, we synthesized some cystine-containing pentapeptide derivatives with some variability on the third position side chain residue (flipping the stereochemistry on the terminal portion and amidating the terminal carboxylic acid functionality) and tested their incorporation into *M. smegmatis* cells. Both altering the stereochemistry and amidating the carboxylic acid residue caused incorporation levels to drop to half the level of Penta LD Cystine's, which is the probe that most closely matches the natural peptidoglycan structure. This data indicates that small alterations to the terminal portion of the third position residue do not completely inhibit crosslinking, corresponding to enzymatic flexibility at this position.

To facilitate the testing of this peptide library, we decided to switch from working in *M. smegmatis* to working in another mDAP containing organism, *L. casei*, which can be more easily grown and produces more homogenous cell populations. Labeling experiments with both tetra and pentapeptides indicated that both Ldts and PBPs in *L. casei* show a preference for the cystine mDAP mimic over the lysine variant. The distinction is more drastic than that observed in *M. smegmatis* during previous assays with Tetra LD Cystine labeling at about twice the level of Tetra Lysine, and the difference even greater between the two pentapeptides.

We also tested the effects of stereochemical changes to the peptide side chain and backbone and how that impacted crosslinking in *L. casei.* Altering the stereochemistry of the side chain of the third residue caused a dramatic decline in labeling, indicating that the Ldts of *L. casei* show a strong preference for the natural substrate. Amazingly, altering the stereochemistry of the terminal alanine residue in the peptide backbone had only a slight effect on labeling levels. The apparent incorporation of Tetra LD Cystine L Ala was a surprising result. Perhaps *L. casei* is less selective than other species when incorporating peptides into its cell wall. Another possible

explanation for the high labeling levels of Tetra LD Cystine L Ala could be due to contamination in the peptide stock or an error when synthesizing the probe (accidental include of D-alanine instead of L-alanine). Further testing will be done to better understand this result.

Another interesting finding common to this series of experiments was the relatively high signal given by heat killed cells. Probe incorporation is thought to occur via a metabolic process, so it was expected that incorporation levels would be low for all heat killed conditions as cells would not be growing and remodeling their peptidoglycan and taking in these molecules. A possible explanation for this phenomenon is a non-specific binding interaction between the peptides and the heat killed cells. The higher labeling levels could also be attributed to not allowing the cells to cool sufficiently before inoculating with the fluorescent probe. This could lead to an artificially high signal because increased fluidity in the cells' outer layers could cause the probes to become trapped in there. A final potential explanation for the high labeling levels in the heat killed cells could be that the cells are being fixed with formaldehyde too quickly. Letting the cells sit in PBS before fixing them could allow for probes stuck nonspecifically to be removed, helping obtain a more accurate measurement for cellular fluorescence levels. The question of what is causing the high fluorescence levels in heat killed cells is still being investigated.

Conclusion:

The overall goal of this work was to develop a library of fluorescently-conjugated molecules that mimic the naturally occurring stem peptides of bacteria which could be used to study crosslinking *in vivo*. By building an assortment of tetrapeptides and pentapeptides, we were able to develop molecules which could be used monitor Ldt activity *in vivo*. Furthermore, by synthesizing similar molecules with slight variations, we were able to determine that amidation of key sites can play a major role in dictating biosynthetic activity. Lastly, an ongoing project involved the synthesis of a cystine-based stem peptide which mimics mDAP at the third position, allowing for more accurate study of the biosynthetic pathways in the large class of bacteria which contain this non-canonical amino acid in their peptidoglycan.

Acknowledgements:

I would like to thank Dr. Marcos Pires for his guidance and leadership over the past three and a half years, as well as for extending the opportunity to become involved with such an exciting and relevant project. I would also like to thank Dr. Jon Fura, Dr. Mary Sabulski, and Sean Pidgeon for their endless help and wisdom throughout the course of the project. Finally, I would like to thank GELH and BDSI for helping to fund the summer research components of the project.

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