Regulation of cell wall biosynthesis in *Bacillus subtilis*

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Objective
The aim of this project is to advance our understanding of the molecular basis for various problems associated with the growth and division of bacterial cells, and how these processes are affected by environmental conditions. Cell shape and cell division are the processes that are majorly determined by localized synthesis of cell wall. We focused on the main aspects of cell wall synthesis, which includes cytoskeletal proteins that provide the spatial signals for synthesis and the catalytic enzymes that make wall precursors and assemble them into new wall material. Cell wall synthesis is also modulated according to the growth state of the cell (e.g. during growth, division, sporulation and competence), and we studied the specialized proteins associated with these processes and their regulation.

Introduction
The shape of Gram positive bacteria is maintained by the cell wall peptidoglycan (PG), a three dimensional meshwork composed of N-acetyl glucosamine and N-acetyl muramic acid which are linked by β-1, 4 peptide cross-linkages. Synthesis and chemical composition of PG are well understood but the structure of the sacculus and the mechanisms controlling its growth is unclear (Brown, Kysela, & Brun, 2011). The machinery that localizes the synthesis of this macromolecular structure dictates the shape of an organism. It is currently assumed that the actin-like MreB proteins form filamentous helical structures along the membrane, which direct cell wall growth by positioning multi-enzyme complexes that mediate sidewall elongation (Kawai, Asai, & Errington, 2009). The peptidoglycan elongation machinery (PGEM) responsible for rod-shaped growth is composed of synthetic enzymes known as penicillin binding proteins (PBPs) and conserved membrane proteins (MreC, MreD, RodA, RodZ). These proteins interact with MreB (Mayer & Amann, 2009), (van den Ent, Amos & Löwe, 2001). Disruption of MreB filaments produces round cells (Kawai, Daniel, & Errington, 2009), (Garner et al., 2011). During exponential growth, MreB proteins did not form helical structures. Instead, together with other morphogenetic factors, they assembled into discrete patches that moved progressively along peripheral tracks perpendicular to the cell axis. Patch motility was largely powered by cell wall synthesis, and MreB polymers restricted diffusion of patch components in the membrane and oriented patch motion. (Domínguez-Escobar et al., 2011).

Peptidoglycan precursors are synthesized inside the cytoplasm as disaccharides. Then they attach to a pentapeptide chain along with a lipid tail (UDP, a 55 carbon chain with a phosphodiester link to the disaccharide). This membrane bound molecule is then translocated by a transporter (probably FtsW for cell division and RodA for cell elongation) from the cytoplasmic side of the membrane to the extracellular surface. Once translocated the PBPs are able to act on these precursors and link them into the cell wall by the action of transglycosylation where they link the sugars to form long chains, up to 300 units in length in the case of B. subtilis and transpeptidases where they crosslink the sugar chains through the pentapeptide portion by using the energy from the D-ala - D-ala bond. These activities seem to be coordinated through the action of the cytoskeleton (MreB-like proteins) or FtsZ so that the "assembly" activity is restricted to specific zones on the cell surface. Through the assembly process it is clear that the cell is able to make new cell wall, however, it is not clear how this is directed so as to enable cell enlargement in a specific direction (elongation of the rod) or division (formation of the septal PG). It was presumed that there must be specific "synthetic complexes" that perform these actions and these have dedicated PBPs specialized for that particular role.
Coordinated synthesis alone is however insufficient to permit cell growth as the cell wall in Gram positives is a substantial, presumably multilayered structure, with new wall material made on the inner surface and old wall hydrolyzed from the exterior surface. In order to operate this efficiently there should be some form of regulated degradation of the PG so as to remove old wall and allow the new wall to become stress bearing, the new wall having been made in a form that can “stretch” and so allow the cell to enlarge. This model of cell growth then requires that the action of the autolytic enzymes to be coordinated in some way. Perturbation of this "coordination" resulting in the loss of cell morphology, and probably viability as well. A collection of mutants have been isolated with cell morphological changes that would be consistent with the loss of wall synthesis coordination - these include MreB - like proteins, RodA, specific Pbps and most interestingly genes involved in the synthesis of a second polymeric wall component, the teichoic acids. Mutations in the genes of the first three proteins can be explained to some degree by having a major role in the synthetic process; however mutation in teichoic acids are not so easily explained (Kawai et al, 2009).

Penicillin-binding proteins (PBPs) are a group of proteins that are characterized by their binding affinity for penicillin. Nearly all beta-lactam antibiotics bind to PBPs and as a result they prevent the cell wall synthesis in the bacterium. There are varieties of PBPs found in different organisms and are present in different numbers, and have varied affinities for penicillin. They can be found as both membrane-bound and cytoplasmic proteins. The PBPs...
are usually broadly classified into high-molecular-weight (HMW) and low-molecular-weight (LMW) categories (Basu, Chattopadhyay, Kundu, & Chakrabarti, 1992). In terms of function all PBPs are involved in the final stages of the synthesis of peptidoglycan, which is the major component of bacterial cell walls. Bacterial cell wall synthesis is essential to growth, cell division and maintaining the cellular structure in bacteria. Inhibition of PBPs leads to irregularities in cell wall structure such as elongation, loss of selective permeability, and eventual cell death and lysis. PBPs have been shown to catalyze a number of reactions involved in the process of synthesizing cross-linked peptidoglycan from lipid intermediates and mediating the removal of D-alanine from the precursor of peptidoglycan. Purified enzymes have been shown to catalyze the D-alanine carboxypeptidase, peptidoglycan transpeptidase, and peptidoglycan endopeptidase. The enzyme has a penicillin-insensitive transglycosylase N-terminal domain (involved in formation of linear glycan strands) and a penicillin-sensitive transpeptidase C-terminal domain (involved in cross-linking of the peptide subunits) and the serine at the active site is conserved in all members of the PBP family (Basu et al., 1992). PBPs bind to β-lactam antibiotics because they are similar in chemical structure to the modular pieces that form the peptidoglycan (Nguyen-Distèche, Leyh-Bouille, & Ghuysen, 1982). When they bind to penicillin, the β-lactam amide bond is ruptured to form a covalent bond with the serine residue at the PBPs active site. This is an irreversible reaction and inactivates the enzyme. There has been a great deal of research into PBPs because of their role in antibiotics and resistance. Bacterial cell wall synthesis and the role of PBPs in its synthesis is a very good target for drugs of selective toxicity because the metabolic pathways and enzymes are unique to bacteria (Chambers, 1999). Resistance to antibiotics has come about through overproduction of PBPs and formation of PBPs that have low affinity for penicillins (among other mechanisms such as lactamase production). Research on PBPs has led to the discovery of new semi-synthetic β-lactams, wherein altering the side-chains on the original penicillin molecule has increased the affinity of PBPs for penicillin, and, thus, increased effectiveness in bacteria with developing resistance. β-lactamases are enzymes produced by some bacteria and are responsible for their resistance to β-lactam antibiotics like penicillins, cephamycins, and carbapenems (ertapenem). (Cephalosporins are relatively resistant to beta-lactamase.) These antibiotics have a common element in their molecular structure: a four-atom ring known as a beta-lactam. The lactamase enzyme breaks that ring open, deactivating the molecule's antibacterial properties (Kawai, Asai, et al., 2009).

Materials and methods

Bacterial strains

A wild type *Bacillus subtilis* 168 CA strain was used in this study. An *E. coli* strain BL21 was used to transform *LytC* and *CwlA* plasmids for the induction study to over express them to generate antibodies. These plasmids were prepared at Centre for Bacterial Cell Biology, University of Newcastle upon Tyne (R. Daniel, unpublished). Various single, double, triple and quadruple Pbp mutants of wild type 168 CA were used in this study to see the effects of various antibiotics on a particular Pbps at MIC.

Preparation of DNA (PCR grade)

The strains were grown up to an OD Α₆₀₀ of ~3.0 in 10 ml PAB (Penicillin Activity Broth). The cells were harvested and washed in TES and resuspended in 0.5 mL TES in 2 ml screw capped Appendorf. 25 µL lysozyme and 5µL RNase were added to digest cell wall and cleave RNA. The tube was then incubated for 15-30 min at 37°C. After that 50 µL pronase, 30 µL sarkosyl was added to cause immediate lysis and incubated for 30 min at 37°C. Then it
was treated by 600 µL CHCl₃ and vortex for ~20 sec, and placed on ice for 30 min. The vortexing was repeated several times during the 30 min incubation. Then it was microfuged for 5 min to separate phases. The aqueous phase was re-extracted as described above. The tube was filled with absolute ethanol and inverted several times until the DNA formed a small clump. It was again microfuged for 5-10 seconds to bring the clump to the bottom of the tube. The supernatant was discarded and the pellet was gently rinsed with 70% ethanol. The pellet was dried briefly in vacuo and then the DNA was re-dissolved in the 100 µL TE buffer. At the end it was heated at 65°C for 10 minutes to make soluble and stored at 4°C. The concentration of DNA was estimated by running an ethidium bromide stained agarose gel and also by using Nano-drop (J Errington, 1984).

Preparation of DNA (transformation grade)
The strains were streaked on a NA plate from the glycerol stock. The plate was incubated overnight at 37°C. Next morning a small loop of colony was inoculated into 2.5 ml PAB in a capped tube and incubated for 3-4 h at 37°C in the tube shaker. After 3-4 hours the cells were in the late exponential growth phase. Then 2.5 ml SSC was added to the culture and the diluted culture was then transferred to a centrifuge tube and cells were pelleted down in bench centrifuge. It was re-suspended in 1 ml SSC and 0.1 mL of lysozyme solution was added afterwards. The tube was incubated in a shaker at 37°C until the cells appear to have lysed. Then 1 ml 4 M NaCl was added to the tube and the suspension was filtered through a sterile 0.45 µ Millipore filter. The DNA solution was freeze-dried at -20°C for later use (Ward & Zahler, 1973).

Plasmid purification
An overnight culture (5 mL) was prepared in LB from which the plasmid had to be purified. The culture was centrifuged and the bacterial cell pellet was resuspended in 250 µL Buffer P1 which was supplemented by RNase and transfer to an appendorf tube. After resuspension of the pellet 350 µL of Buffer P2 was added and gently mixed the tube by inverting 4–6 times. When the solution started becoming clear (after around 5 min) 350 µL of buffer N3 was added and gently mixed the tube by inverting 4–6 times. Proper mixing gave a cloudy solution. The solution was centrifuged for 10 min at 13,000 rpm in a table-top centrifuge machine. The supernatant obtained from centrifugation was then applied to the QIAprep spin column by decanting gently. It was again centrifuged for 45 seconds and the flow-through was discarded. The QIAprep spin column was washed by adding 0.5 ml Buffer PB and centrifuged for 45 seconds and the flow-through was discarded. The QIAprep spin column was washed by adding 0.75 ml PE Buffer and centrifuged for 45 seconds. The flow-through was discarded and centrifuged for an additional minute to remove residual wash buffer. The QIAprep column is then placed in a clean 1.5 ml appendorf tube. 50 µL sterile water placed in the spin center to elute the plasmid. The column was kept on the bench for 1 min, and then centrifuged for an additional minute (adapted from Qiagen).

Transformation (E.coli)
To transform E. coli the following protocol was used.

Preparation of competent cells (BL21)
BL21 was grew in 30 ml LB medium with 10 mM of MgSO4.7H2O and incubated at 37°C. When an OD₆₀₀ nm reached about 0.3-0.4, cool the flask on ice for 10 min, then spin down the culture, discard the supernatant and re-suspend the cells in pre-cold 15 mL of 100 mM CaCl₂. After that the suspensions was on held on ice for an hour and then spun down again,
discard the supernatant and re-suspend the cells in 3 ml of 50 mM CaCl2, 10% glycerol. Again hold on ice for 20 min and then aliquot in 100 µl aliquots and snap frozeed in liquid nitrogen and stored at -80 degrees.

**Transformation with the plasmids (LytC and CwlA)**

Using the cells made above, one aliquot was defrosted on ice and 1 µl of the plasmid was added in a sterile tube with 50 µl of competent cells. It was held on ice for 45 min, heat shocked the cells for 90 seconds at 42°C and the returned on ice for about 2 min, then 250 µL of LB broth was added to the appendorf and incubated at 30°C for an hour. From the appendorf 100 µl was plated out onto a kanamycin plate and incubated overnight at 30°C.

From the overnight plates two individual colonies of each construct were taken and streaked out on a nutrient agar plate and incubated overnight at 30°C. Untransformed BL21 strain was also streaked out. The next morning, a 25 mL culture in a 250 ml flask with 100 µg/mL kanamycin was inoculated. The cultures were kept at 37°C until they reached an OD 0.4, then 0.5 mM IPTG was to the culture and continued to grow. After every 30 minutes of intervals 1 ml of sample was harvested form each culture. Each sample was then processed for SDS-PAGE analysis of the total proteome.

**Transformation (B. subtilis)**

*B. subtilis* 168 CA was streaked out on a nutrient agar plate and grew overnight at 37°C. The following morning two tubes were prepared with 5 mL of PTM (pre-transformation medium) in each of them. It was heavily inoculated with a big loop full of cells from the overnight plate, and the other one was inoculate with rather fewer cells. The culture was kept in a shaker at 37°C and OD<sub>600</sub> was followed. After several hours the cultures reached stationary phase (A<sub>600</sub> = 3.0). At that point the cells began to be competent. During the growth in PTM, TM (Transformation Medium) was prepared. Two tubes with 1 mL of TM medium was prepared and kept at 37°C for pre-warming (Errington, 1984).

After an OD of one of the cultures reached an OD 3.0, 0.1 mL of the culture and DNA to be transformed was added to each of the tubes containing TM. The tubes were incubated in a shaker for 30-90 min at 37°C. After the incubation 0.1 mL of the transformed culture was transferred onto a NA plate with an appropriate antibiotic. A control plate with only competent cells and only DNA was also prepared to test the competent cells and sterility of DNA. These plates were then incubated for overnight 37°C.

**Expression**

Single colony of *E. coli* BL21 (transformed with LytC and CwlA) were inoculated in 2 L flask with 500 mL LB medium. After an OD 0.4, the cultures were induced by adding 1 mM IPTG as a final concentration. Samples were taken at different time points (0.5, 1, 2 and 3 hours).

**Lysis**

The harvested samples were then centrifuged to pellet and snap freezeedd in liquid nitrogen to analyze it later. Next day cell pellets were defrosted and resuspended in 200 µL of loading buffer. Samples were lysed by using sonication (15 seconds x 3 rounds) to allow all the intracellular proteins to come out into the suspension. After the sonication, protein samples were denatured at 80°C for 10 minutes and loaded on the gel. The amount of sample was
back calculated according to the optical density of the original culture before loaded on to the gels.

**Affinity Purification**

The culture of desired strain was grown and induced as described above and harvested after two hours in two big centrifuge tubes. The sample was centrifuged at 4000 rpm for 10 minutes and the pellet was snap frozen in liquid nitrogen to analyze later. Next day the pellets were defrosted and resuspended in 10 mL 8 M Urea, 20 mM sodium phosphate buffer and 200 mM sodium chloride buffer. Both resuspended cultures were merged into one centrifuge tube (40 mL) which gave me the total volume of 20 mL. The culture was sonicated for 3 burst of 30 seconds and kept on ice in between to keep the temperature down. Whole cell lysate was then centrifuged at 20,000 rpm for 20 minutes to pellet down all the big lumps of cell wall, DNA and membrane proteins, leaving only low molecular weight proteins in the supernatant. The supernatant was filtered through 0.8 and 0.45 µ Millipore filters. At this time point the column and column material was prepared as described above. The column was washed twice with sterile water and equilibrated by passing the urea buffer (5 mL * 4 times) through it. The whole cell lysate was passed through the column containing immobilized nickel ions, which binds the polyhistidine tag. Usually the recombinant protein is the only protein in the mixture with this affinity, which aided in separation. Thus by immobilizing nickel on a resin, specifically only his-tagged protein bound. Since the protein was the only component with a His-tag, all other proteins passed through the column, and left the His-tagged protein bound to the resin. After that the column was repeatedly washed by urea buffer to remove other non-specific binding of proteins. The protein of interest which was bound to nickel ions washed with the low concentration of imidazole, which competes with the polyhis-tag for binding to the nickel column. Low concentration of imidazole only allowed the washing out of loosely, non-specifically bound and high molecular weight proteins which also stuck into the column. After an imidazole washing the column was again washed with urea buffer. And then finally the protein is released from the column in a process called elution, by using 100 mM EDTA. The protein of interest is now the major protein component in the eluted mixture, and can easily be separated from any minor unwanted contaminants by a second step of purification, such as size exclusion chromatography (Continuous Elution Electrophoresis). These samples were then run into the SDS-PAGE to verify the purity and the concentrations of fractions of protein containing aliquots. At the end Acetone precipitation was used to precipitate the protein and get a dry pellet to send it off for the antibody generation.

**SDS-PAGE**

SDS-PAGE is a technique for separating proteins based on their ability to move within an electrical current, which is a function of the length of their polypeptide chains or of their molecular weight. This is achieved by adding SDS detergent to remove secondary and tertiary protein structures and to maintain the proteins as polypeptide chains. The SDS coats the proteins, mostly proportional to their molecular weight, and confers the same negative electrical charge across all proteins in the sample. Glycosylated proteins may not migrate at their expected molecular weight since their migration is based more on the mass of their polypeptide chains, and not the sugars that are attached to it because they do not bind to SDS in a defined way (Sambrook, etal, 1989).
The average apparent molecular weights of the protein bands in the BenchMark Pre-Stained Protein Ladder are shown below in Table 1. The DNA ladder was separated on Novex 4-20% Tris-Glycine gel.

Table 1. Prestained protein ladder from BenchMark

<table>
<thead>
<tr>
<th>Band No.</th>
<th>Average Apparent Molecular Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>~180 kDa</td>
</tr>
<tr>
<td>2</td>
<td>~115 kDa</td>
</tr>
<tr>
<td>3</td>
<td>~82 kDa</td>
</tr>
<tr>
<td>4</td>
<td>~64 kDa</td>
</tr>
<tr>
<td>5</td>
<td>~49 kDa</td>
</tr>
<tr>
<td>6</td>
<td>~37 kDa</td>
</tr>
<tr>
<td>7</td>
<td>~26 kDa</td>
</tr>
<tr>
<td>8</td>
<td>~19 kDa</td>
</tr>
<tr>
<td>9</td>
<td>~15 kDa</td>
</tr>
<tr>
<td>10</td>
<td>~6 kDa</td>
</tr>
</tbody>
</table>

*Orientation band (pink in color)

**Detection**

The polyhistidine-tag can also be used in detection of the protein via anti-polyhistidine-tag antibodies or alternatively by in-gel staining (SDS-PAGE) with fluorescent probes bearing metal ions. This can be useful in sub cellular localization, ELISA, western blotting or other immuno-analytical methods.

**Western Blotting**

There are different basic methods for transferring proteins from gel to membrane - capillary blotting, diffusion blotting, and electro blotting.

**Electrophoresis**

The proteins from SDS gel were then transferred to nitrocellulose sheets as follows. The physical assembly used is shown diagrammatically in Figure 2. A sheet of nitrocellulose (0.45 μm pore size in roll form, Millipore) was briefly wetted with water and laid on a scouring pad (Scotch-Brite) which was supported by a stiff plastic grid. An extra layer of blotting paper was used to make sure that the layers do not move during the electrophoresis. The gel to be blotted was put on the nitrocellulose sheet and care was taken to remove all air bubbles. A second pad, blotting paper and plastic grid were added and it was locked to fix all the layers together. The gel was thus firmly and evenly pressed against the nitrocellulose sheet. The assembly was put into an electrophoretic chamber (Bio Rad, Trans Blot™ Cell) with the nitrocellulose sheet facing the cathode. The chamber contained 0.5x NuPAGE MES running buffer and 20% methanol. 25 mA Current was applied for overnight (Towbin, Staehelin, & Gordon, 1979).

**Blotting**

![Blotting diagram](image)
The membrane was carefully removed from the overnight electrophoretic chamber and was transferred into a phosphate buffered saline (1 PBS tablet/100 mL) + 0.1% Tween 20 + 5% dried skimmed milk. The corner of the membrane was cut to know the orientation and then the membrane was put with 20 mL of above blotting mixture. The membrane was kept shaking in the above mixture for an hour to allow blocking of all the non-specific binding sites of the membrane. After an hour the His-tag antibodies were added (1:1000) to the mixture and further allowed antibodies to bind to the specific binding sites for next hour. The membrane was then washed with PBS + Tween 20: 3x short wash for 5 minutes and 5x long wash for 15 minutes to make sure the all the unbound antibodies is washed out. The membrane was then kept in PBS + Tween 20 and dried skimmed milk mixture for 15 minutes to block again, and then the secondary specific anti-mouse antibody was added (1:10,000) into the mixture and kept shaking for another hour to allow binding of secondary antibodies to the primary antibodies. Finally the membrane was washed with PBST several times to remove all unbound antibodies from the membrane which would interfere with the detection.

The detection was performed as per instructions in the manual of the “Amersham ECL prime western blotting detection reagent”.

Figure: 2. Western blot of CwlA protein. The bottom most bands are CwlA proteins bound with various concentrations of secondary antibiotics.

The antibodies were raised against the purified CwlA protein and were checked by using it in Western blotting. Last protein is the CwlA monomer while the others on top of it are dimmers, trimers and tetramers.

**MIC determination**

Minimum inhibitory concentration of various β-lactam antibiotics were determined by titrating various concentrations on the exponentially growing *B. subtilis* culture. To determine that first of all we have prepared the list of all the relevant antibiotics and other compounds
which can inhibits the action of bacterial $\beta$-lactamases such as tazobactam. By using this compound we can determined if the recovered culture is effect of $\beta$-lactamase activity or not. Glass test tubes with 2 mL of PAB medium were inoculated by an exponentially growing culture of OD 0.4 (in PAB) which gave a culture of 4 mL with the final OD$_{600}$ of 0.2. The 2 mL PAB medium in the tubes were already mixed with double amount of antibiotics, ranging from 100 µg/mL to 0.0032 µg/mL, which reached to the required concentration after diluting it with 2 mL of growing culture. These cultures were then incubated in the 30ºC room and samples were taken after every 30 min starting from $T_0$. These samples were collected in the eppendorf which contained 3 µL of Bocillin dye. (It has been used to determine the penicillin-binding protein profiles of a number of different bacteria types. It is a new, sensitive, rapid, and nonradioactive method for the detection and study of penicillin-binding proteins (PBPs). This method allowed rapid detection of 2 to 4 ng of the protein with the aid of a FluorImager) (Zhao, Meier, Kahl, Gee, & Blaszczak, 1999). After harvesting these samples were rapidly freezed in liquid nitrogen and then stored at -20ºC. Next day the samples were lysed using sonication for 15 sec x 3 times. These samples were then loaded into the SDS PAGE gel with the running buffer and protein ladder. After 50 min the gel assembly opened and the gel was scanned in the fluoroimager. The pictures obtained from this imager are shown in the results section along with their MIC growth curves to correlate the growth, MIC and Pbp profile.

Results

Determination of MICs and binding affinity of B-lactams

Various $\beta$-lactamase sensitive and non-sensitive antibiotics were used to see the effects on Pbp profile in \textit{B. subtilis} at MIC. Previous studies showed that the not all the Pbps are essential for the growth. However it has not been shown exactly which Pbps and what minimum concentration is required for growth. In this study we have determined the MIC for almost 20 antibiotics in PAB (Penicillin Assay Broth) medium and at MIC we have shown the Pbp profile and their effects on the growth of \textit{B. subtilis} 168 CA. On the basis of the results obtained we have divided the antibiotics in 3 different groups to simplify our understanding. We have picked approximate MICs for these antibiotics from these experiments and the same experiments were then repeated on that MIC to detect the exact MIC in given conditions. In these experiments we have also studied the Pbp profile by using the Bocillin dye to correlate the growth at MIC and effect of various antibiotics on the Pbp profile.

![Figure 4. Mechanism of $\beta$-lactamase to deactivate the $\beta$-lactam ring of the antibiotics](image-url)
Table 2: The effect of various β-lactam antibiotics

<table>
<thead>
<tr>
<th>No.</th>
<th>Antibiotic</th>
<th>MIC (µg/ml)</th>
<th>Kill effect</th>
<th>Effect on the basis of highest concentration in the experiment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PonA</td>
<td>PbpA</td>
</tr>
<tr>
<td>1</td>
<td>Cefamandole</td>
<td>0.0064 - 0.032</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>2</td>
<td>Cefazolin</td>
<td>0.032 - 16</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>3</td>
<td>Cefmetazole</td>
<td>0.16 - 0.8</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>4</td>
<td>Cephalaxin</td>
<td>0.5 - 1</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>Cloxacillin</td>
<td>0.8 - 0.16</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>Oxacillin</td>
<td>0.032 - 0.16</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>7</td>
<td>Amoxicillin</td>
<td>0.8 - 4</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>8</td>
<td>Azlocillin</td>
<td>0.16 - 0.8</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>9</td>
<td>Cefapirin</td>
<td>0.16 - 0.8</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
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<td>Cephalothin</td>
<td>0.032 - 0.16</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>11</td>
<td>Mecillinam</td>
<td>0.16 - 0.8</td>
<td>Full</td>
<td>+</td>
</tr>
<tr>
<td>12</td>
<td>Pencillin</td>
<td>0.8 - 0.16</td>
<td>Full</td>
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<tr>
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<td>Cefotaxime</td>
<td>0.4 - 2</td>
<td>Titration effect</td>
<td>+</td>
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<td>Cefazidime</td>
<td>0.4 - 2</td>
<td>Titration effect</td>
<td>+</td>
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<td>0.4 - 2</td>
<td>Titration effect</td>
<td>+</td>
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<tr>
<td>16</td>
<td>Carbencillin</td>
<td>0.8 - 4</td>
<td>β-lactamase sensitive</td>
<td>+</td>
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<td>17</td>
<td>Cefoxitin</td>
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<td>β-lactamase sensitive</td>
<td>+</td>
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<td>+</td>
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<td>β-lactamase sensitive</td>
<td>+</td>
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<td>-</td>
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<td>7-aminocephalosporanic acid</td>
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<td>-</td>
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<td>-</td>
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<td>24</td>
<td>Cephalosporin C zinc Pract</td>
<td>ND&lt;sup&gt;2&lt;/sup&gt;, 100</td>
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<td>25</td>
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<td>ND&lt;sup&gt;2&lt;/sup&gt;, 100</td>
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<tr>
<td>26</td>
<td>D-minus-penicillamine</td>
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<tr>
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<td>31</td>
<td>Dicloxacillin</td>
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</table>

ND<sup>2</sup> = No effect detected until the concentration mentioned after the comma.
The flouroimages are shown next to the growth curves (in most cases) with antibiotics treatment at MIC. The box in the right indicates the various concentrations used in the experiment and in the left side there are various Pbps indicated on the gel image. All of them have compared to the WT.

In case of Cefotaxime it seems that at the higher concentration PonA and PbpF are completely gone, while Pbp A, B, C and 5 are unaffected. On the other hand in Cefoperozone PbpC seems to be induced more than a WT which might indicate some transcriptional regulation or making up the work load of other Pbp which has been lost.

![6-10% gradient gel](image)

(a)

Figure 5 (a). In this figure the curves are showing the profiles of the antibiotic effects and the gel picture showing the Pbp profiles of the same samples at various time points. Same legend would be valid for all the similar figures which are listed below i.e. (b), (c), (d), (e), (f), (g), (h), (i), (j), (k), (l), (m), (n), (o), (p)
Ceftazidine and Moxalactam also had a titration effect on the growth and Pbp profile also looked the same as Cefotaxime. Moreover, there was no Pbp which was specifically targeted by both of them. However, Pbp5 seems to gone in Moxalactam unlike Ceftazidine. There growth resumed as the concentration has gradually decreased.

Even after 100 µg/mL these two antibiotics hardly had any effect on the Pb profile.
7-aminoccephalosporanic acid also did not have considerable effect on the Pbp profile of the organism while Cefmetazole was deadly on Pbps and the growth. Even on the lowest concentration of 0.032 µg/mL it was showing less than half the growth compared to the WT.

Dicloxacillin and Ceftiraxone were very lethal to the organism and apart from Pbp5 they have wiped out almost all the Pbps which resulted in the total retardation in the growth.
These two hardly had any effect on the Pbp profile.

Cephalomamine did not have much effect on the specific antibiotics, however, Amoxicillin was also lethal to the cells as it has wied out almost all the Pbps.
Azlocillin and Cefapirin were also very lethal to the cells as they have also wiped out almost all the important Pbps.

Phenolxymethylpenicillin and Carbenicillin also wiped out most of the Pbps but even then they were able to resume their growth (probably) due to the β-lactamase activity.
At low concentration of Cloxacillin it was lethal to the cell and as the concentration decreased the β-lactamase activity resumed the growth.

At low concentration of Ticarcillin it was lethal to the cell and as the concentration decreased the β-lactamase activity resumed the growth.
Cefamandole was extremely lethal to the cells that’s why they wiped out almost all the Pbps from the cells. However, Cefoxitin was able to recover (probably) due to the β-lactamase activity.
Cefazolin was also very deadly to the cells as it wiped out all the important Pbps and allowed no cells to grow more than half the final OD (at 0.0064) compared to the WT.
Cefsulodin and Oxacillin both were similarly lethal to the cells and did not allow the culture to resume growth until the low concentration of 0.16 µg/mL.

Cephalexin was very interesting one from the above – as it was very lethal up to a certain point (4 µg/mL) and then as the concentration went down suddenly the culture resumed their growth and went to the OD$_{600}$ close to the WT.
Strong and clear example of β-lactamase sensitive antibiotic. At the concentration of 20 µg/mL almost all the Pbps were gone while the growth did not get affected drastically.

Effect of clavulanic acid and tazobactam
In some of our previous experiments after the antibiotic treatment we have observed that one of the Pbp was over expressed which was very unlikely because we had expected that all the Pbps should be wiped or at least partially. However, when we looked the picture in higher resolution gel we saw that the band is most probably PbpC. To confirm this result we had thought of using a mutant strain of WT in which PbpC has been deleted.
For the experiment I have used Wild type *B. subtilis* 168 CA and its mutant 168CA Δ*pbpC*. The result expected here would very simple that if still see that same over expressed band in WT and also in Δ*pbpC* that would mean that it was not PbpC and something else. However, if we see the gel below carefully, it is clear that the band we were looking for (PbpC) is again over expressed in the WT and not present in the mutant strain.

Apparently PbpC has been over expressed when we treat the cells with clavulanic acid and tazobactam and couple of other antibiotics. It may have some transcriptional regulation or also possibility that PbpC start taking over the duties of one of the lost Pbp in the neighborhood.

![Image of gel showing overexpression of PbpC](image)

**Figure 15.** *B. subtilis* 168 CA treated with clavulonic acid and tazobactam which resulted in the over expression of PbpC.

![Image of phosphoimage](image)

**Figure 16.** Phosphoimage of the *B. subtilis* lysate. Which shows that PbpC is over expressed when its treated with tazobactam.
**Purification of Autolytic enzymes and antibody production**

LytC and CwlA are the amidases. These proteins were tagged by Histidine, purified by using several steps as described below and used to generate antibodies. These constructs were needed to be tested for their overexpression ability and yield. They were transformed into *E. coli* strain BL21 and then induced by adding IPTG. The purification methodology were optimized and also checked for the solubility. The ultimate objective was to have these enzymes in a form that can be used for antibody generation, biochemical characterization and crystallization.

**CwlA purification**

BL21 transformed with CwlA was induced by adding 1mM IPTG and the lysate was allowed to flow through the resin saturated with nickel sulphate. The column was then washed by pouring different concentrations of Imidazole and was finally eluted by using 100 mM EDTA. In the gel picture below it is very evident that the protein of interest was over-expressed and eluted with EDTA. This was further confirmed by MS spectroscopy.
Figure 8. His-tagged CwlA eluted in 100 mM EDTA by using Ni-column. CwlA has a molecular weight around 30 kDa and it is nicely separated from adjacent protein bands which helped in getting pure fractions of CwlA by CEE.

Protein sample eluted in EDTA was then treated with acetone (figure: 4) which was able to remove some of the low molecular weight unwanted proteins. Sample obtained by acetone precipitation was run through continuous elution electrophoresis (figure: 5) where all of proteins got separated nicely on the basis of their molecular weight and size.

Figure 9 & 10. CwlA after acetone precipitation. Where all the low molecular weight proteins got removed. (9) Sample obtained from acetone precipitation was then processed in Continues Elution Electrophoresis (CEE). This sample was again cross checked by running into SDS PAGE and Nano drop.
Mass Spectrometry

After getting the nice and clear bands on the SDS-PAGE it was necessary to double check the purity of the protein of interest. So we run the sample through MS spectroscopy. The reason for this experiment was that the protein did not run as per its molecular weight size. There can be several explanations for proteins not running at the expected place in the protein gels; e.g. Proteins changes there confirmation depending on the pH, temperature and the presence of SDS in the sample buffer. The ladder we used is pre-stained protein ladder which also has a margin of error as stated in the Invitrogen manual.

The SDS PAGE gel containing nice and clear band was cut using scalper knife and send for the MS analysis. The results obtained are posted below which confirmed that the samples contained pure LytC and CwlA.
Figure 12. Mass spectrometry results of the CwlA protein.

Figure 13. Mass spectrometry results of the CwlA protein.
**PbpA and PbpH interaction**

To identify the potential interactions between PbpA and PbpH we have tried to create two constructs with his-tag attached to them. Tagging histidine would have given us a way to pull most proteins which are interacting with PbpA and PbpH. Below is the schematics of the gene fragment to be constructed.

Primers for *pbpA*, *Pxyl* his-tag and its homologous upstream fragment were constructed using Clone Manager software®. These fragments were amplified by using polymerase chain reaction (PCR) which was further purified and treated with restriction enzymes to digest the ends. After that they were checked on the agarose gel and then ligated using ligase enzyme. Picture below is showing the PCR products.

![PCR Products](image.png)

Figure 14. These PCR product were correct however the ligation of the Xba1 end were having some complications.

**Discussion**

In order to understand the mechanism of the cell wall biosynthesis at the molecular level we wanted to study the effects of various β-lactam antibiotics on the Pbp profile in *Bacillus subtilis*. Pbps are one of the most important tools in the cell wall biosynthesis in *B. subtilis*. They link the precursors, translocated from the cytoplasm, into the cell wall by the action of transglycosylation and transpeptidase. We also tried treating the culture with other compounds like tazobactam instead of β-lactam antibiotics to see how they affect the Pbp profile in time in the cells. We were also trying to correlate this Pbp study with how they interact with their surrounding environment, especially with neighboring Pbp and other proteins. We came up with the plan in which we decided to his-tag the PbpA and PbpH, as they were the redundant pair, and pull down the proteins interesting with them.

In order to study the effects of various β-lactam antibiotics on the Pbp profile, we initially determined the closest MIC value of various antibiotics. After that we have done various growth experiments in which we have treated an exponentially growing culture with various antibiotic concentrations, which are listed in table 2. We have also tried some concentrations above and below the MIC value and then cells were harvested at regular intervals to determine the Pbp profile using the penicillin binding dye. The dye was already in the sampling tube and then the cells were immediately mixed, centrifuged and stored at -
20°C to analyze it later. Next day after breaking open the cells it was ran through the SDS protein gel to see which Pbps were inhibited and which once are still remaining. What we had expected out of this experiment that particular antibiotics will target and inhibit a specific Pbp but what we observed was very interesting. We have observed that the effects of the antibiotics were not the same in terms of Pbp inhibition. It was very divert, however when we looked the data carefully we saw that antibiotics can be divided into four groups depending upon their more of actions. The first one was with Cefamandole, Cefazolin, Cefmetazole, Cephalexin, Cloxacillin, Oxacillin, Amoxicillin, Azlocillin, Cefapirin, Cephalothin, Mecillinam, Pipercillin, was able to inhibit almost all the major types of the Pbps within a time frame of seconds which resulted in the simple lysis of the culture. Second group was with Cefotaxime, Ceftazidine, Moxalactam had a titration effect where the growth was retarding proportionally to the concentration of the antibiotics added. The third group had (Carbenicillin, Cefoxitin, Penicillin G, Phenoxymethylpenicillin, Ticarcillin) was even more interesting since they first show retarding in growth and then after a while they resumed their growth. The possible explanation for this growth patters could be that these particular antibiotics were β-lactamase sensitive which was secreted by the cells and this enzyme breaks that ring open, deactivating the molecule's antibacterial properties. The last group or so to say the fourth group had (7‐amincephalosporanic acid, 7‐aminodesacetoxycephalosporanic acid, Cephalomannine, Cephalosporin C zinc Pract, Cephradine, D‐minus‐penicillamine) no effect on Pbp profile, hence no effect on the growth. However, as a concluding sentence it would be a nice experiment if one could try using various mutants of Pbps and then do these experiments find out more details about their mode of action and mode of killing. Especially if we want to know the specific targets.

We had expected to see the clear effects of particular antibiotics on a specific Pbp target however we managed to connect some parts of the puzzle. The mode of action of these antibiotics is still not clear, however, people claim that it not just one target, it is a combination things which is happening in side the cells. As it was suggested above, these types of experiments can be repeated in the strains with no redundant Pbps and so allow the identification of the important targets of these antibiotics.

Also the mode of action for Clav and Tazo in modulating the expression of PBP C – something that is not seen for all B-lactams – is this related to another aspect of the antibiotics action?

References


