Studies towards the development of a DART-MS based assay to study the kinetics of PgaB, a de-N-acetylase essential for biofilm formation

by

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A thesis submitted in conformity with the requirements for the degree of Masters of Science
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Abstract

Bacterial biofilms are surface-attached microbial communities embedded in an extracellular matrix. The extracellular matrix of many infectious bacterial strains contains partially de-\textit{N}-acetylated \( \beta \)-(1,6)-\textit{N}-acetylglucosamine polymers (dPNAG). In \textit{E. coli}, the CE4 deacetylase PgaB catalyzes partial de-\textit{N}-acetylation of PNAG, which is essential for biofilm formation. PgaB selectively de-\textit{N}-acetylates the third position of a synthetic PNAG pentasaccharide in vitro, however, the observed catalytic efficiency is poor with a \( k_{\text{cat}}/K_M \) of 0.25 M\textsuperscript{-1}s\textsuperscript{-1}. We hypothesized that PgaB has higher catalytic activity with the natural PNAG polymer. Using DART-MS an assay has been developed to study the kinetics of PgaB by monitoring the release of acetate. The DART-MS based assay provided comparable kinetic information as the fluorescamine assay upon analysis of a simple reaction mixture of PgaB and PNAG oligomers, showing that DART-MS is a promising tool to monitor such reactions. However, it was not useful for a multi-enzyme reaction due to strong matrix effects.
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1 Introduction

1.1 Biofilm

1.1.1 What are biofilms?

Bacteria exist in two growth modes; nonsessile planktonic mode and sessile biofilm mode. The planktonic mode is the state in which bacteria exist suspended in a media, not attached to a surface. The biofilm mode is the state in which bacteria exist in a surface-attached community adhered to a biotic or abiotic surface, as well as to other microorganism of either the same species or different species. Most bacteria tend to occur in the latter mode; planktonic growth mode rarely exists in nature.¹

Biofilms are defined as irreversibly surface-attached microbial communities in which the bacteria/microorganisms cells (~15% by volume) are entrapped in a self-produced matrix (~85% by volume) that is made up of nucleic acid molecules, proteins and exopolysaccharides.²,³ Biofilms maintain optimal conditions for bacteria to grow by regulating pH conditions and redox potential. They contain water channels that are thought to facilitate the diffusion and circulation of nutrients and oxygen.⁴ Furthermore, sessile bacteria in biofilms are usually more tolerant to stress, and killing conditions, such as antibiotics and attacks by the host immune system, as a result they cause chronic infections.

1.1.2 Biofilm formation

Biofilm formation is multi-stage process that starts with attachment followed by maturation and then the cycle comes full circle by dispersion (Fig. 1). In mature biofilms all these stages might be present at the same time. The cells undergo a significant change in physiology throughout the entire process.⁵
1.1.2.1 Attachment

This stage is divided into two sub-stages: 1) reversible and 2) irreversible attachment. When the planktonic bacteria come in contact with a surface they initially adhere reversibly. If this surface is suitable for them, they start to irreversibly attach to the surface.\textsuperscript{5}

The following factors affect whether the attachment will take place or not, and control the rate and the extent to which the colonization occurs:

1. **Substratum**

   The more hydrophobic the surface is the stronger the attachment. It was shown that strongly adherent bacterial strains colonized a rough surface better and more easily than a smooth surface.\textsuperscript{1,5} Hydrophobic surfaces interact better with the hydrophobic cell surface, and this strong interaction allows the cell to overcome any repulsive electrostatic barrier between the polar components on the cell surface and the polar components on the surface.\textsuperscript{1,5} Additionally, if the surface is conditioned/coated by a film from the aqueous medium where it exists, such as blood, saliva and respiratory secretions, the attachment of the bacteria to the surface is enhanced.\textsuperscript{1,5}

2. **Environment**

   Biofilms seem to form better in high-shear environments, since the rapid flow assists in the strong attachment to the surface. Biofilms formed in low-shear environments are weak and
break easily. Furthermore, the conditions of the environment such as pH, ionic strength, nutrient concentrations and temperature affect the extent and rate of colonization. The nutrients concentrations are directly proportional to the strength of the attachment. Also, the presence of divalent cations increases the attachment since they enhance the interactions between the surface and the cell surface.\textsuperscript{3,1}

3. **Cell**

The characteristics of the bacterial cell play a role in the attachment to the surface and the strength of that attachment. Motile cells attach better than nonmotile ones since the nonmotile cells cannot recognize empty spaces to colonize so biofilm development is slow. Furthermore, the polysaccharides on the surface of the cell play a role in the attachment due to their interactions with the surface.\textsuperscript{5,1}

If all the conditions are suitable, the reversible attachment leads to a stronger irreversible one, and changes in regulation of many genes occur. It was shown via protein analysis that there is a significant difference in the cell physiology between the reversible and irreversible stages.\textsuperscript{5}

1.1.2.2 **Maturation**

In this stage cell clusters develop fully into a mature biofilm. The first sub-stage is maturation-1. This stage is characterized by the activation of biofilm regulatory systems that trigger the gene expression pattern changes to start the process of matrix production and biofilm development.\textsuperscript{5}

In the second sub-stage: maturation-2, the biofilm development is complete; the biofilm reaches its maximum thickness. In this stage, protein patterns are significantly different from planktonic bacteria; the regulation of more than 50\% of the proteins examined is affected. There is also a six fold or greater change of protein regulation in maturation-2 than in maturation-1.\textsuperscript{5} This shows how the physiology of the cells in the biofilm changes drastically throughout the process of biofilm development.

1.1.2.3 **Dispersion**

This is the last stage that brings the biofilm formation to a full circle by dispersing motile bacterial cells to populate other surfaces. The change in cell physiology is demonstrated in this
stage as well. The protein patterns from this stage resemble those of the planktonic bacteria and
differ substantially from those of the cells in maturation-2 stage.\textsuperscript{5}

LysR, RNA-binding protein CsrA (carbon storage regulator A) has been shown to regulate
biofilm dispersion in \textit{E. coli}. CsrA is regulated by the availability of nutrients; ie carbon flux, in
the environment.\textsuperscript{6,7} Some strains of \textit{E. coli} also activate a dispersin enzyme that would interact
with adhesive components of the biofilm to promote dispersion.\textsuperscript{7}

1.1.3 Biofilm and Infection

Biofilms are responsible for 65\% of chronic infections as well as medical-device-related
infections.\textsuperscript{8} Some of the biofilm-associated pathogenic bacteria genera are \textit{Escherichia,
Legionella, Staphyloccouccus, Streptococcus} and \textit{Vibrio}. It was observed that antibiotic therapy
ends the apparent symptoms of an infection but does not cure the infection. This caused a
dilemma that was not solved until the switch in paradigm about the cause of infection, biofilms
not planktonic bacteria, took place. The antibiotics used killed the planktonic bacteria in the
blood but they were not successful in killing the biofilm. This is why in many infections, the
symptoms came back because the biofilm would continue to disperse cells into the host. Besides
the fact that biofilms are resistant to antibiotics, they release antigens and trigger the production
of antibodies, which can cause the immune system to damage to nearby tissues without clearing
the biofilm.\textsuperscript{2,4,9}

There are multiple mechanisms of how biofilms resist antimicrobial agents and spread disease\textsuperscript{3};

1. Slow diffusion of antimicrobial agent across the biofilm

The matrix acts as a diffusion barrier for these agents either physically by slowing their
diffusion rate or chemically by reacting with them. In both cases, the agents do not end up
reaching the cells and as a result are not able to kill the baceteria.

2. Slow growth rate in the biofilm

The slower the growth rate of bacteria, the slower the ability of the cells to take in
antimicrobial agents. It was found that the slowest growing \textit{E. coli} strain (in biofilm) was
the most resistant to cetrimide.

3. Physiological changes in the cells
Cells under stress, nutrient limitation, oxygen depletion, or build-up of toxic metabolites do not uptake the antibiotics efficiently due to their changed physiology. So sometimes those cells would be on the surface to prevent or slow down the uptake of antimicrobial agents.

4. **Conjugation/Gene transfer**

Conjugation is the process of plasmid transfer, which favourably takes place in biofilms due to the close proximity of cells. This leads to the rapid transfer of resistance to antimicrobials between different species in the biofilm.

The last stage in biofilm formation and development is dispersion in which aggregates or cells are detached from the biofilm. Usually the released cells carry the same phenotype as the biofilm, such as resistance to antimicrobial agents, and when they populate a new surface in the body a secondary antibiotic resistant-infection results. This is how some bloodstream and urinary tract infections are formed, from aggregates generated from the medical-devices biofilms.¹,³

1.1.4 dPNAG

An essential feature of the biofilm, which is about 85% by volume, is the self-produced matrix that allows the cells to adhere to the surface and to each other. It is made up of different macromolecules: proteins, nucleic acids, humic substances, lipids and exopolysaccharides (EPS). The main component of the matrix is the EPS, they make up about 50-90% of the matrix, and the amount differs depending on the species and the biofilm.⁴,¹⁰ EPS play a major role in the attachment stage and provide protection to the biofilm in various ways; first, they contribute to the antimicrobial resistance. EPS act as a diffusion barrier to the antimicrobial agents. Furthermore, they stabilize the biofilm allowing it to withstand environmental stresses. Also, they are highly hydrated because they bind water in their structures via hydrogen bonding. This allows EPS to prevent the biofilm from dissociating and withstand humid conditions. Part of this hydrated network, specifically the outermost layer, sometimes would dry out to form a hardened shell around the biofilm, protecting it. EPS’s properties and characteristics vary depending on the environment where the biofilm exists to protect and stabilize it.¹,¹⁰

Different species and different strains of bacteria produce a variety of EPS, such as dPNAG, cellulose, colonic acid, alginate, Pel and Psl, which are all essential in maintaining the structure and integrity of the biofilm in their respective species.¹¹,¹² dPNAG, partially de-N-acetylated
polymeric β-1,6-N-acetyl-D-glucosamine (poly-β-1,6-GlcNAc) is the focus of this thesis (Fig. 2b). It is found in numerous biofilms of medically important bacterial strains. It was found in the biofilms of *S. epidermidis*, *S. aureus*, *E. coli*, *Yersinia pestis*, *Yersinia pseudotuberculosis*, *Yersinia enterocolitica*, *Acinetobacter baumannii*, *Actinobacillus actinomycetemcomitans*, *Actinobacillus pleuropneumoniae*, *Burkholderia* species, *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Xanthomonas axonopodis*, *Pseudomonas fluorescens* and others.\(^{13,14,15,16,17,18,19,20}\) dPNAG is essential for biofilm formation and the adhesion processes in these bacteria. When dPNAG-producing bacteria are grown in the presence of dispersin B (DspB), an enzyme that specifically hydrolyzes the glycosidic linkages in dPNAG, biofilm formation is inhibited. The result of DspB biofilm inhibition is similar to knocking out the enzymes involved in the biosynthesis of dPNAG.\(^{13}\) Another study showed that when dPNAG was degraded by metaperiodate (HIO\(_4\)), the biofilm dissociated.\(^{15}\) These studies show that dPNAG is essential for biofilm formation and adhesion. Biofilm disruption is not observed when the formation of other polysaccharides such as colanic acid or cellulose is prevented. dPNAG plays a role in the attachment stage, and the transition from the temporary to permanent attachment.\(^{17}\) It was found that under shaking conditions, dPNAG was located at the cell poles through which the initial attachment to the surface takes place.\(^{17}\)

dPNAG biosynthesis is a synthase-dependent secretion system, which is one of the three mechanisms bacteria use to synthesize and export polysaccharides, the other two involve the Wzx/Wzy and ATP-binding cassette pathways.\(^{21}\) dPNAG biosynthesis and export is controlled by a four-gene operon; *pgaABCD* operon in *E. coli* and *icaADBC* operon in *S. epidermidis*.\(^{15}\) The *pgaABCD* operon codes for four proteins; PgaA, PgaB, PgaC, and PgaD. PgaC and PgaD are cytoplasmic membrane proteins, which are essential for PNAG synthesis while PgaA and PgaB are outer membrane proteins, which are essential for PNAG export (Fig. 2).\(^{17,22}\) PgaCD start the polymerization of PNAG from UDP-N-acetylglucosamine (GlcNAc) donor and translocate it across the inner membrane from the cytoplasm to the periplasm.\(^{17,22}\) PgaB is a lipoprotein associated with the inner leaflet of the outer membrane that partially de-N-acetylates the PNAG polymer; 3-5% of PNAG is de-N-acetylated.\(^{11,17}\) PgaB then sends dPNAG off to PgaA to be exported across the outer membrane.\(^{17,22}\) The glucosamine units in the polymer are essential for its export through PgaA; when PgaB is deleted the polymer is retained in the periplasm and is not exported.\(^{17,22}\) The positively charged units mediate the interaction of the
polymer with the bacterial surface as well, via electrostatic interactions between them and the negatively charged components on the cell surface.\textsuperscript{17,22}

The dPNAG biosynthesis is tightly regulated via the expression regulation of \textit{pgaABCD} operon. The LysR family DNA-binding protein NhaR is required for the biosynthesis to start since it activates \textit{pgaABCD} transcription. This protein is regulated by pH and concentrations of sodium

Figure 2: a) Synthesis, modification and export of dPNAG via pga machinery, b) dPNAG, partially de-N-acetylated polymeric $\beta$-1,6-$N$-acetyl-D-glucosamine (poly-$\beta$-1,6-GlcNAc).\textsuperscript{60} (Figure 2a is adapted from ref. 60).

The dPNAG biosynthesis is tightly regulated via the expression regulation of \textit{pgaABCD} operon. The LysR family DNA-binding protein NhaR is required for the biosynthesis to start since it activates \textit{pgaABCD} transcription. This protein is regulated by pH and concentrations of sodium
ions in the environment. In addition, CsrA negatively regulates dPNAG biosynthesis. When it is deleted, dPNAG is overproduced and a significant increase in biofilm formation is observed. CsrA represses the synthesis of dPNAG by blocking the ribosome access to the pgaA Shine-Dalgarano sequence and by destabilizing pgaABCD transcript. Furthermore, the second messenger bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) controls the levels of PgaD post-transcriptionally, PgaD is degraded in the absence of c-di-GMP and is unstable at low cellular concentrations of c-di-GMP. Therefore, c-di-GMP acts as a regulator for the PNAG biosynthesis since the reduction or absence of c-di-GMP can cause the Pga machinery to shut off and stop PNAG biosynthesis.

1.1.5 Pga machinery

1.1.5.1 PgaCD

PgaC is a cytoplasmic membrane protein located in the inner membrane. It is a processive β-glycosyltransferase (GT), which is a member of the GT-2 family. It polymerizes PNAG from UDP-GlcNAc. It is made up of two domains A and B with active sites in both for the sugar polymerization reaction. The transmembrane region mediates the translocation of the polymer across the membrane to the periplasm. PgaD is a small integral inner membrane protein that assists PgaC in the polymerization and translocation processes. The $K_M$ of PgaCD for UDP-GlcNAc is 270 µM, which lies within the range of reported cellular UDP-GlcNAc concentrations in E. coli, which means that UDP-GlcNAc does not act as a limiting factor under conditions favouring EPS synthesis and biofilm formation.
It was recently found that c-di-GMP is essential for dPNAG polymerization and translocation across the inner membrane because it allosterically activates the PgaCD complex. It mediates the interaction between the two proteins, stabilizes and activates them. It binds to PgaCD complex with high affinity ($K_{\text{act}}=62\ \text{nM}$) and increases the rate of polymerization of the complex. The suggested model is that c-di-GMP binds to both proteins, inducing a conformational change that allows the integration of the transmembrane domains of both proteins; two transmembrane helices of PgaD integrate into the core of the transmembrane domain formed by PgaC. This allows the formation of a stable, active and secretion-competent heterodimeric complex, since a pore is opened in the cytoplasmic membrane to allow for the translocation of PNAG to the periplasm (Fig. 3). Thus, c-di-GMP allows both proteins to form an active biosynthetic complex enabling them to perform their function.\textsuperscript{23}

Figure 3: Schematic diagram of the effect of c-di-GMP on the interaction between PgaC and PgaD.\textsuperscript{23} (Figure is adapted from ref. 23).
1.1.5.2 PgaA

PgaA is an outer-membrane protein that is essential for the secretion of dPNAG. It is a two-domain protein. The C-terminal is a 16-stranded β-barrel domain that acts as the porin for exporting dPNAG, while the N-terminal periplasmic domain carries multiple copies of the tettratricopeptide repeat (TPR) motif that is believed to play a major role in protein-protein and protein-dPNAG interactions.\(^{17,27,28}\)

It was recently found that a cluster of negatively charged residues close to the periplasm inside the PgaA barrel lumen is essential for dPNAG secretion. It is suggested that there are two sets of electrostatic interactions; one that acts as the driving force for passage of dPNAG through the pore, and another that facilitates the secretion process. The former is between the positively charged units in the polymer and the negatively charged residues in the porin, while the latter is between the positively charged units interact with the negatively charged components on the cell surface.\(^{11}\) This finding explains why when PgaB is deleted, dPNAG accumulates in the periplasm and its secretion is inhibited. Since PgaB is essential for the change of the charge on the polymer by de-N-acetylating it forming the primary amine which then gets protonated. The TPR motifs are essential for the function of PgaA, because the β-barrel of PgaA alone was not functional and was not able to export the polymer.\(^{11}\) It was found that the TPR domain of PgaA and PgaB directly interact, so it is suggested that interaction is a key for the polymer to be brought in proximity to the porin prior to export.\(^{11}\)

1.1.5.3 PgaB

PgaB is a two-domain outer membrane lipoprotein that partially de-N-acetylates PNAG in the periplasm. Without the partial de-N-acetylation of PNAG, PgaA cannot export the polymer and PNAG accumulates in the periplasm. This shows how crucial PgaB is to the polymer export and secretion processes.\(^{17,22}\) The N-terminal domain is a member of carbohydrate esterase family 4 (CE4). CE4 enzymes are known to deacetylate O- and N-acetylated polysaccharides such as chitin, peptidoglycan and acetylxyan.\(^{29,30}\) The C-terminal domain (residues 310-672) shows similarity to glycoside hydrolase 18 family (GH18) and GH20. The GH families bind and hydrolyze GlcNAc substrates.\(^{30,31}\) The N-terminal domain is responsible for the de-N-acetylation activity while the exact role of the C-terminal hydrolase is still not completely understood. The
PgaB C-terminal domain, however, facilitates the de-N-acetylation of the polymer since the N-terminal domain is enzymatically less active without C-terminal domain.  

The PgaB C-terminal domain lacks a catalytic residue in the active site that is found in most, if not all the GH18 and GH20 members; Glu140 in AMCase (acidic mammalian chitinase) or Glu184 in DspB. Even though it has another residue, Asp467 that could be a replacement, it is buried and is not accessible. Furthermore, PgaB did not hydrolyze para-nitophenyl GlcNAc, para-nitrophenyl is an artificial substrate with which glycoside hydrolases typically show activity. Therefore, it is thought that this domain may be an inactive hydrolase. It was shown that it has preference for GlcNH$_3^+$ binding over GlcNAc, which correlates with the stimulation study that shows that it preferentially binds dPNAG over PNAG. Therefore, it is suggested that de-N-acetylation takes place first then the C-terminal domain mediates its interaction with PgaA. PNAG is believed to bind to the cleft between the N- and C- terminal domains. Structural studies of the PNAG oligomers suggest that they have a tendency to adopt stacked or helical structures which lead to the prediction that the inter-domain cleft binds PNAG in an extended conformation. Therefore, the long polymer might be required for efficient catalysis. This might
explain why PgaB showed poor catalytic efficiency of $k_{cat}/K_{M}=0.25 \pm 0.01 \text{ M}^{-1}\text{s}^{-1}$ with PNAG pentamer which is 2000-fold less efficient than that of $SpPgdA$, a CE4 peptidoglycan deactylase PgdA from $S. pneumoniae$, with $\beta$-(1,4)-(GlcNAc)$_3$. K$_M$ could not be calculated due to the poor solubility of the pentamer, which prevented saturation of PgaB activity, but it is estimated to be greater than 25 mM. 

PgaB is a metal-dependent protein like other CE4 proteins. These enzymes use a divalent metal cofactor to catalyze deacetylation reactions. PgaB has preference for Co$^{2+}$, Ni$^{2+}$ and Fe$^{2+}$. The identity of the endogenous metal that PgaB binds is not known but it is suggested that it depends on the environment the bacteria is in and the availability of metals.

Figure 5: a) Proposed catalytic mechanism of PgaB based on $SpPgdA$ catalytic mechanism, b) Catalytic residues in PgaB active site coordinated to Ni$^{2+}$. (Figure 5a is adapted from ref. 38).
It is suggested that the catalytic mechanism of PgaB is similar to that of SpPgdA. It starts by the binding of the GlcNAc residue to be de-N-acetylated to the active site; the N-acetyl methyl group points towards a hydrophobic pocket (Leu274-Leu291), while the carbonyl and C3 hydroxyl group are coordinated to the metal ion. Then, the water bound to the metal deprotonates Asp115 leading to a nucleophilic attack of the carbonyl resulting in the tetrahedral oxyanion intermediate. The intermediate is stabilized by the metal and amide of Tyr252, then His55 protonates the N-acetyl nitrogen generating a free amine leaving group and the acetate as a by-product (Fig. 5).22,34,38

![Figure 6: PgaB selectively de-N-acetylates the third position of β-(1,6)-GlcNAc pentamer.](image)

Most of the current PgaB studies are conducted with the pentamer because kinetic studies showed that the activity of PgaB increases as the length of the oligomer increases up to the pentamer, and there was not any significant increase with the hexamer. Also, it specifically de-N-acetylates the third position (Fig. 6).22 The assay usually used to study the de-N-acetylation activity of PgaB is a discontinuous fluorescamine assay. It detects the amount of free primary amines in solution because fluorescamine selectively reacts with them to generate a fluorophore that can be monitored at \( \lambda_{\text{ex}} = 360 \text{ nm} \) and \( \lambda_{\text{em}} = 460 \text{ nm} \) (Fig. 7).22,38,39
1.1.5.4 Catalysis and Rate constants

PgaB is missing a catalytic Asp residue, which is found in more competent CE4 enzymes. Additionally, PgaB de-N-acetylates 5% of PNAG polymer and when overexpressed the percentage increases to only 22%. However, the observed \( k_{\text{cat}}/K_{\text{M}} \) (0.25 +/- 0.01 M\(^{-1}\)s\(^{-1}\)) of PgaB with the PNAG pentamer is not explained by the hypothesis that PgaB is a slow enzyme. Since the average metabolic enzyme would have a \( k_{\text{cat}}/K_{\text{M}} \approx 10^5 \text{ M}^{-1}\text{s}^{-1} \), which is 400000 fold more than the observed PgaB \( k_{\text{cat}}/K_{\text{M}} \) with the pentamer. Additionally, relative to other CE4 enzymes PgaB has significantly lower catalytic activity. \( \text{SpPdgA} \) has a \( k_{\text{cat}}/K_{\text{M}} = 150 \text{ M}^{-1}\text{s}^{-1} \) with \( \beta-(1,4)\text{-}(\text{GlcNAc})_3 \), the peptidoglycan deacetylase from \( B. \text{cereus} \) has a \( k_{\text{cat}}/K_{\text{M}} = 21300 \text{ M}^{-1}\text{s}^{-1} \) with \( N\text{-acetylchitooligosaccharides (GlcNAc})_5 \) and chitin deacetylase from \( \text{Collectotrichum lindemuthianum} \) has a \( k_{\text{cat}}/K_{\text{M}} = 89000 \text{ M}^{-1}\text{s}^{-1} \) with \( \beta-(1,4)\text{-}(\text{GlcNAc})_5 \) which are 2000, 85200, and 356000 fold larger than that of PgaB with the PNAG pentamer, respectively.

Therefore, we hypothesized that despite the attempts to characterize PgaB as a slow enzyme, the observed catalytic efficiency with the PNAG pentamer is not reflective of the true activity of PgaB in vivo. We believe that more kinetic studies are needed with PgaB and the natural substrate, which may allow us to understand how PgaB functions in vivo.

1.1.6 Analytical techniques to detect and quantify acetate

One of the ways to monitor deacetylation reactions, and study the kinetics of PgaB, is to monitor the release of acetate. There are multiple analytical techniques that could be, and have been, used to detect and quantify the amount of acetate in a mixture.
1. **NMR**\(^{42}\): NMR can be used to quantify the amount of acetate in solution by adding an internal standard and by integrating the peaks. However, the limitation with such a technique is that the sample has to be relatively clean, and there cannot be overlap with the acetate peaks in the NMR. Thus, for an enzymatic reaction, NMR would not be the best option since the sample would need to be purified first, to at least remove all the proteins. At that point, it is not clear if there may still be molecules in the reaction mixture that would overlap with acetate peaks.

2. **High Pressure Liquid Chromatography (HPLC)**\(^{43}\): HPLC has been used to detect and quantify acetate from biological samples. Therefore, it would be a viable tool to use to study kinetics of PgaB and quantify acetate in an enzymatic reaction. However, conducting kinetics studies, which implies taking multiple time points for multiple reactions with replicates, would be time consuming. In addition, for a complex reaction mixture that contains proteins and sugars a costly column would be required.

3. **Gas chromatography (GC)**\(^{44}\): GC has been used to detect and quantify acetate. However it would not be applicable for enzymatic reactions that are in aqueous solution because water would poorly evaporate in GC experiments.

4. **Coupled-enzyme assays**\(^{45-47}\): Commercial kits are available that can quantify acetate in aqueous solutions. Acetate is detected via a cascade of reactions to produce a NAD\(^+\) or another chromophore that can be quantified by absorbance or fluorescence. However, an assay mixture that contains proteins may interfere with the signal. Therefore, the sample has to be purified first to remove all competing enzyme activities.

### 1.2 Objectives and Purpose of current study

As discussed, PgaB was found to have a poor catalytic efficiency with the PNAG pentamer with a \(k_{\text{cat}}/K_M\) of 0.25 M\(^{-1}\)s\(^{-1}\).\(^{22}\) There are multiple hypotheses for why this was observed, which are not mutually exclusive; 1) the full length polymer is required in order for PgaB to function efficiently, since one of the suggestions of how the substrate binds to the protein is that it “wraps” around the two domains, 2) the concentrations are different from the \textit{in vivo} concentrations, 3) protein-protein interactions; such as interactions with PgaA and its TPR
domain, or the entire quaternary complex, PgaABCD, are needed so that PgaB functions efficiently.

We decided to tackle the first hypothesis, which is using the natural substrate, the full length PNAG rather than short PNAG oligomers. The reason the full length PNAG was not used before is that the reaction mixture would be complex since the reaction contains impure PgaCD membrane extracts to make the PNAG polymer, UDP-GlcNAc that would be converted to semi-soluble PNAG, PgaB, alkaline phosphatase, cycloserine and c-di-GMP. Monitoring the de-N-acetylation of such a reaction mixture using the fluorescamine assay is almost impossible due to the following problems; 1) semi-soluble polymer, 2) impure membrane extracts and 3) high background signal from all the components of this reaction mixture. Therefore, another assay is needed to monitor the de-N-acetylation reaction and to be able to study the kinetics of PgaB.

We hypothesized that DART-MS would be a promising tool to monitor acetate production in this multi-enzyme system, since no sample purification is required. DART-MS has not been used to detect and quantify acetate before. However, DART-MS produces comparable results to conventional LC-MS on in vivo and in vitro ADME (absorption, distribution, metabolism and excretion) samples with no purification. Additionally, DART-MS was used in the analysis of fatty acid and methyl esters from whole cells and other complex mixtures and materials.48,49 This shows that DART-MS has the potential to be used quantitatively in some biological matrices.

The use of DART-MS would introduce a new methodology to study enzyme kinetics in a more native environment. Additionally, it would provide us with a great tool to further study the Pga machinery and the biosynthesis of dPNAG.
Chapter 2

2 DART-MS

2.1 DART-MS

DART-MS is a technique used to analyze samples in ambient conditions at atmospheric pressure. The key characteristic of ambient mass spectrometry methods is that they require minimum sample preparation. The sample does not need to be pre-treated or purified before being analyzed.\textsuperscript{49–51} DART-MS uses dry heated helium gas excited species (He\(^\ast\)) that have formed in a plasma discharge to start a cascade of gas-phase reactions leading to the ionization of the analyte via charge transfer and Penning ionization methods. In order for this to take place the analyte has to be in the gas phase, therefore He is heated so it "wipes off" the analyte at the surface and gets it into the gas phase. The He gas is usually heated in the range of 100-550 °C depending on the identity of the analyte. The temperature affects significantly the quality of the spectrum obtained.\textsuperscript{49,50,52,53}

DART is able to analyze high and low polarity compounds but not ionic compounds, under negative or positive ion modes. It is also unable to analyze high molecular weight compounds; the range of the compounds it is able to analyze is between m/z 50 to m/z 3500 depending on their thermal stability and volatility.\textsuperscript{49,54} Furthermore, the matrix makes a difference, therefore, running standards in different matrices is necessary to compare the matrix effects as those can affect the signal significantly.\textsuperscript{48} Additionally, the microenvironment affects the spectrum, including the composition of the laboratory atmosphere, solvent vapours, humidity or unwanted gaseous components. Thus, the sensitivity of the DART can be a limitation to this technique.\textsuperscript{49,55}

Figure 8 illustrates the DART-MS, the first component is the DART ionization source, followed by the reaction zone, and finally the mass spectrometer that analyzes the ionized analyte.

He gas goes through a needle electrode to the first compartment where it gets exposed to discharge currents on the order of 2 mA and at a gas temperature of 50-60 °C, this is known as corona-to-glow discharge. This process produces cations, electrons and excited He atoms (He\(^\ast\)). These components go through a second compartment where the cations and electrons are
removed from this mixture to leave only the excited He atoms. He gas gets heated to the set
temperature before it enters the reaction zone, which is the area between the DART ion source
and the inlet to the mass spectrometer, where the analyte is placed.\textsuperscript{49,50,52,53} He* starts a cascade
of gas-phase reactions and the analyte is removed from the surface and gets it into the gas
phase.\textsuperscript{49,56} The ionization processes are based on the Penning ionization and charge transfer
methods and they differ according to the mode of the DART source, either positive-ion or
negative-ion mode.\textsuperscript{49,50}

\begin{center}
Figure 8: Top is a picture of the DART-MS, bottom is a schematic diagram of DART-MS. a)
DART ionization source through which He passes and He excited species (He*) are formed and
heated. b) reaction zone hot stream of He* evaporate the analyte and it starts a cascade of gas-phase
reactions that ionizes the analyte. c) mass spectrometer where the ionized analyte gets analyzed.
\end{center}

In positive-ion mode these are the reactions that take place
\begin{itemize}
  \item He* $+ N_2 \rightarrow$ He $+ N_2^+$ $+ e^-$
  \item He* $+ M \rightarrow$ He $+ M^+$ $+ e^-$
  \item N$^+$ $+ N_2$ $+ N_2 \rightarrow$ N$_4^+$ $+ N_2$
\end{itemize}
• \( \text{N}_4^+ + \text{H}_2\text{O} \rightarrow 2\text{N}_2 + \text{H}_2\text{O}^+ \)
• \( \text{H}_2\text{O}^+ + \text{H}_2\text{O} \rightarrow \text{H}_3\text{O}^+ + \text{OH}^- \)
• \( \text{H}_3\text{O}^+ + n\text{H}_2\text{O} \rightarrow [(\text{H}_2\text{O})_n +\text{H}]^+ \)
• \( \text{M} + [(\text{H}_2\text{O})_n +\text{H}]^+ \rightarrow [\text{M} + \text{H}]^+ + n\text{H}_2\text{O} \)

**Direct charge transfer**
• \( \text{N}_4^+ + \text{M} \rightarrow 2\text{N}_2 + \text{M}^+ \)
• \( \text{O}_2^+ + \text{M} \rightarrow \text{O}_2 + \text{M}^+ \)
• \( \text{NO}^+ + \text{M} \rightarrow \text{NO} + \text{M}^+ \)

While in the negative-ion mode different sets of reactions take place
• \( \text{He}^* + \text{N}_{\text{gas}} \rightarrow \text{He} + \text{N}_{\text{gas}}^{+} + e^- \)  \( \text{N}_{\text{gas}} \) \( \)is neutral species in gas phase
• \( \text{He}^* + \text{N}_{\text{surf}} \rightarrow \text{He} + \text{N}_{\text{surf}}^{+} + e^- \) \( \text{N}_{\text{surf}} \) surface
• \( \text{O}_2 + e^- \rightarrow \text{O}_2^- \)
• \( \text{O}_2^- + \text{M} \rightarrow [\text{M} + \text{O}_2]^- \)
• \( [\text{M} + \text{O}_2].^- \rightarrow \text{M}^+ + \text{O}_2 \)
• Depending on the nature of the analyte the following reactions can also take place,
  ▪ Direct electron capture by the analyte: \( \text{M}^+ + e^- \rightarrow \text{M}^- \)
  ▪ Dissociative electron capture: \( \text{MX} + e^- \rightarrow \text{M}^- + \text{X}^- \)
  ▪ De-protonation by dissociation or reaction with a base: \( \text{MH} \rightarrow [\text{M}-\text{H}]^- + \text{H}^+ \)
  ▪ Anion attachment: \( \text{M} + \text{X}^- \rightarrow [\text{it M}+\text{X}]^- \)

One of the advantages of DART is that fragmentation is not a problem because it works at ambient pressure. The ions are thermalized immediately due to high collisions rates and as a result fragmentation is reduced.49

### 2.2 Results and Discussion

#### 2.2.1 Optimizing conditions

The following parameters affect the DART-MS spectra and signal detection and had to be optimized:

1. **Vapur Interface**: The Vapur interface was used because it enhances the transfer of ionized analyte from the reaction zone to the mass spectrometer. It resembles a jet-
separator removing light species such as He and air and as a result reducing the background signal. However, using it affected the signal significantly and the signal was not reproducible. This may be due to the vapor interface pumping away light molecules and it might be pumping away some of the acetate in the sample intended for analysis. Therefore, we decided not to use the Vapur interface since the spectra were significantly better and were more reproducible in the absence of the Vapur interface.

2. **Temperature:** Using samples with no proteins or oligomers in the initial optimization conditions, the following temperatures were tested 100, 150, 200, 250, 300, 400 and 500 °C. 300 °C gave the best signal and was used in future studies. However, once samples with PgaB and oligomers were run at 300 °C the sugar and the protein were observed to decompose on the capillary tube. Therefore, the same range of temperatures was reevaluated while changing other parameters; such as volume of sample and speed of rail until good signal was observed at 150 °C. 150 °C was low to evaporate the analyte as the signal intensity was low, but 200 °C proved to be the “sweet spot”; it was high enough to evaporate the analyte and low enough not to decompose the sugars or the proteins. 250 °C was also tested but sugar decomposition was observed.

3. **Volume of Sample:** The volume was optimized so that the smallest amount of assay mixture was required for the assay. Different volumes were evaluated; 1, 2, 3, 5 µL. 3 µL was used at the very beginning however, as other parameters were optimized we were able to use 2 µL of sample. 1 µL was too little to result in a reproducible good signal.

4. **Speed of the rail:** The rail controls how fast the capillaries move past the ionization source. First the rail was only used to set the distance of the sample from the DART source. However, as the number of samples increased and the number of replicates required increased the rail had to be used for efficiency. The slowest speed of 0.2 mm/sec resulted in the best signal and faster speeds did not result in a reproducible signal.

5. **Distance of the DART source from the mass spectrometer:** The distance that resulted in the best signal was 5 cm. Larger distances resulted in a poor signal likely because these resulted in most of the He* being lost to the atmosphere. Shorter than 5 cm, specifically 3.7 cm, which was the distance set on the instrument, resulted in a poor signal.
6. **Time for the sample to dry on the capillary tube before analysis:** Initially it was not clear that the sampling time makes a difference in the spectra. Once this was observed range of time periods was tested and examined; 1, 2, 3, 5 minutes; good spectra of samples with no sugar or proteins were obtained with a 5 minute drying time. However as the complexity of the reaction mixture increased, 5 minutes was not sufficient to dry the samples completely. So another range of time periods was tested; 5, 8, 9 and 10 minutes. 10 minutes was the shortest time it required for the samples to dry completely and resulted in reproducible spectra.

7. **Quadrupole voltage:** The voltage was set at the beginning at the default of 300 V and it worked well. Unfortunately, one day a high background signal was observed and was not reduced until the voltage increased, we are still unsure of the cause of this change in the instrumental settings. The quadrupole voltage controls the range of ions that go through to the detector. The smaller the voltage the lighter the ions that are detected. 

When this problem was observed, a range of voltages was tested; 300, 350, 400, 450, 500, 550, 600, and 650 were examined. 300-450 V had a strong signal for 59 m/z (mass of acetate) and 73 m/z (mass of propionate) but also a high background signal. 550-650 V had poor signal for the desired masses. 500 V was the sweet spot that resulted in the best signal for 59 m/z and 73 m/z with minimum background signal.

8. **Concentration of internal standard (propionate):** The concentration of propionate had to be adjusted depending on the acetate concentration expected, because of signal suppression. If the propionate signal is too high, it suppresses the acetate signal and vice versa.

The optimization of these parameters was based on observation, trial and error and going back and forth between different parameters. When one parameter was optimized, another one, which was already optimized, had to be re-optimized. Also, when the sample matrix changed some parameters had to be re-optimized.
2.2.2 DART is able to detect a range of acetate concentrations

DART-MS conditions were optimized to ensure that different concentrations of acetate could be detected quantitatively and reproducibly (section 4.4). Propionate was selected as the internal standard because; 1) its mass (73) does not overlap with background peaks unlike other isotopologues of acetic acids, 2) it has similar, if not identical, chemical properties as acetate and 3) it is cheap and commercially available. A range of acetate concentrations from 2.5 µM to 1 mM was analyzed by DART-MS and quantified using propionate, 50 µM (Fig. 9). The experiment was repeated on another day to ensure that the results were reproducible. It has been shown previously that the spectra could be affected significantly as the microenvironment conditions around DART-MS change.\(^4^9\) The standard curve revealed a linear relationship between the acetate signal and the acetate concentration of the sample. Additionally, there was no significant difference between the results over two day, which shows that this technique is reproducible.

![Graph showing standard curve of acetate in phosphate buffer ranging between 1000 and 2.5 µM. DART temp: 150 °C, [propionate]= 50 µM, R^2 = 0.998 (day 1), 0.997 (day 2), slope = 18.1 mM^-1 (day 1), 16.1 mM^-1 (day 2).](image)

The next goal was to introduce PgaB and PNAG oligomers to the standards to ensure that the linear relationship between acetate ion signal and acetate concentration was maintained in a more complex mixture. This involved a re-optimization of several parameters involved in the experiment. The standard curve over a range of acetate concentrations showed that DART-MS could be used quantitatively to detect acetate in a mixture containing PgaB and PNAG oligomers.
The range of acetate concentrations was changed because under those conditions (20 
µM PgaB and 20 mM PNAG oligomers) the maximum amount of acetate expected was 2 mM. 
Since the first time point is after 2 hours, it was estimated that 0.1 mM of acetate would be 
produced by then. Thus, the previous range would not be applicable for the conditions of the 
experiments to be conducted.

2.2.3 Fluorescamine vs DART-MS

After showing that DART-MS can be a good tool to monitor de-acetylation, it had to be 
compared against an assay already being used to do so: a fluorescamine assay. PgaB (20 µM) 
was incubated with PNAG oligomers (20 mM) and propionate as an internal standard (0.5 mM) 
at 37 °C. Time points over 52 h were analyzed with DART and fluorescamine assays (Fig. 11). 
The rate of de-N-acetylation of PNAG oligomers by PgaB from both assays was within the same 
order of magnitude; rate from DART was 2.2 x 10^{-8} M/s ± 2 x 10^{-9} M/s and that from 
fluorescamine was 3.4 x 10^{-8} M/s ± 2 x 10^{-9} M/s. Taking into consideration that these two assays 
each detect a different molecule, such similar derived rates show that DART is a viable tool to 
monitor a de-N-acetylation reaction.

Figure 10: Standard curve of acetate in reaction mixture of PgaB and PNAG oligomers ranging 
between 0.1 and 2 mM, [propionate] = 0.5 mM, [PgaB] = 20 µM, [PNAG oligomers] = 20 mM  R^2 = 
0.974 (day 1), 0.995 (day 2), slope = 2.16 mM^{-1} (day 1), 2.44 mM^{-1} (day 2).
2.2.4 Using DART to detect de-N-acetylation of polymer

After demonstrating that DART could be used to monitor the de-N-acetylation of PNAG oligomers, we hypothesized that it may be able to monitor the de-N-acetylation of full length PNAG. However, first it was necessary to demonstrate that acetate standards could still be detected quantitatively in the complex reaction mixture of PgaCD membrane extracts and UDP-GlcNAc/polymer. The curve of the standards with PgaCD membrane extracts (1 mg/ml), UDP-GlcNAc (20 mM) and different concentrations of acetate ranging between 0 to 2 mM resulted in a straight line reproducing the linear relationship observed previously (Fig. 12).
Three sets of reactions were set up in duplicate containing PgaCD (1.5 mg/ml), UDPGlcNAc/PNAG oligomers (20 mM), and PgaB (20 µM). Time points over 32 h were collected and analyzed using DART (Fig. 13). The resulting data show large error associated with each time point, which shows that DART-based assay is unable to reliably detect acetate in such a complex reaction mixture. This is due to the strong matrix effect that developed as the complexity of the reaction mixture increased.

To illustrate the matrix effect, three samples of increasing complexity were analyzed by DART. The ion-extracted chromatogram of the sample with no proteins or sugar shows sharp distinct peaks of the same height (Fig. 14). However, when the protein and PNAG oligomers were added the peaks were no longer as reproducible within the same sample but the internal standard compensated for this variability. When membrane extracts were added and the growing polymer was present in the reaction mixture, distinct peaks were hard to detect, which reflects the strong matrix effect on the signal. Furthermore, despite the reproduced linear relationship in the three different standard curves, the slope was significantly different reflecting the difference in

Figure 13: Concentration of acetate in three sets of reactions over 32 h, all contained [propionate] = 0.5 mM, [PgaB] = 20 µM, [Alkaline phosphatase] = 0.02 units/µl; reaction 1: [PgaCD membrane extracts] = 1mg/ml, [UDPGlcNAc] = 20 mM. reaction 2: [PgaCD membrane extracts] = 1 mg/ml, [GlcNAc oligomers] = 20 mM, reaction 3: [PgaCD membrane extracts] = 5 mg/ml, [UDPGlcNAc] = 20 mM.
sensitivity of DART-MS as the mixture increased in complexity. The slope of the line reflects sensitivity. The steeper the slope the higher the sensitivity of the technique. This means that DART-MS can distinguish between small changes in acetate concentrations reliably. The slope for standards with no proteins or sugar is 18 mM⁻¹, while that for those with PgaB and PNAG oligomers is 2.4 mM⁻¹, and that for those with membrane extracts is 0.77 mM⁻¹. (Fig 9, 10 & 12) This illustrates the drastic change in sensitivity in the technique.

This shows that DART-MS is a good tool to monitor de-N-acetylation reactions in a simple reaction mixture but as the complexity of the mixture increases, this technique fails to detect acetate reliably.

Figure 14: extracted ion chromatogram of acetate for a) a sample with no protein nor sugar, b) a sample with PgaB and PNAG oligomers, and c) a sample with membrane extracts and UDPGlcNAc/polymer.
Chapter 3

3 Conclusion and Future Directions

Our results demonstrate that DART-MS is a useful tool to monitor de-\(N\)-acetylation reactions in simple matrices. It was shown that DART-MS can be used in simple systems since it produced comparable results to the fluorescamine assay with reactions containing PgaB and PNAG oligomers. However, it is not applicable for our complex system due to matrix effects that increase with the complexity of the reaction mixture. It has been shown before that the matrix does affect spectra significantly in DART and it is something that is not easily avoided.\(^{48}\)

However, that leaves us with questions to be answered, therefore, another assay had to be developed to monitor the de-\(N\)-acetylation reaction. We decided to explore an enzyme-coupled assay that detects acetate in solution (Megazyme, Acetic Acid Assay K-ACETRM). The presence of acetate starts a cascade of reactions and eventually produces NAD\(^+\) that can be detected at \(\lambda = 340\) nM (Fig.15).\(^{45-47}\)

![Diagram of reactions](image)

Figure 15: Cascade of reactions that start with acetate and eventually produce NAD\(^+\) that can be detected at \(\lambda = 340\) nM.
In order for this assay to be used, the proteins in the PNAG production and de-N-acetylation reaction mixture had to be removed to avoid any interference with the enzymatic reactions involved in the enzyme-coupled assay. Samples of the polymerization reaction with different acetate concentrations were prepared. They were then subjected to protein extraction by adding chloroform followed by vigorous vortexing. The aqueous layer was extracted and used for analysis. In order to ensure that no acetate was lost in that process, these samples were compared to solutions containing only identical concentrations of acetate. The standard curves of both sets of samples show a linear relationship between the signal and the acetate concentration over a range of 0.1-1.6 mM (Fig. 16). Additionally, there is no significant difference between the two standard lines showing that this necessary sample preparation does not affect the concentration of acetate in the sample. The slopes of the two lines are comparable; 0.207 (samples treated with chloroform), and 0.217 (samples with no protein added), which shows that the sensitivity of the assay was not compromised with the purified samples. Therefore, in the future, this assay will be used to study the kinetics of PgaB with the polymer and compare them to those with the PNAG oligomers. Then, we will study the effect of quaternary complex (PgacC, PgaD, PgaB and TPR in PgaA) on the rate of the de-N-acetylation reaction to study the effects of protein-protein interactions on the catalytic efficiency of PgaB.

![Figure 16: Standard curve of two sets of sample; 1) (triangles) no protein; the samples contained only different concentrations of acetic acid in buffer, 2) (diamonds) chloroform; the samples contained PgaCD (1mg/ml), UDPGlcNAc (20 mM) with different concentrations of acetic acid, then protein was precipitated out and the aqueous layer was analyzed.](image-url)
Chapter 4

4 Experimental

4.1 PNAG oligomers synthesis and purification

An acid reversion reaction was found to efficiently synthesize PNAG oligomers. It involved stirring N-acetylglucosamine in 70% HF, pyridine for 5 days at room temperature. This resulted in different lengths of PNAG oligomers which are then purified using an HPLC size exclusion column (Gilson 321 HPLC pump with a Gilson UV-Vis 156 dual k absorbance detector over Bio-Gel P-4 gel resin)(Fig. 17).^[57]

Fig 17^[57]: a) Acid reversion reaction to synthesize PNAG oligomers b) size exclusion HPLC trace for the different oligomer sizes 1) hexamer, 2) pentamer, 3) tetramer, 4) trimer, and 5) dimer.
4.2 Protein purification

4.2.1 PgaB purification

The procedure described in Little et. al.\textsuperscript{22} was followed. All steps were performed at 4°C, unless otherwise stated. \textit{E. coli} BL21 cells were transformed with pET28-PgaB\textsubscript{22-672}, encoding PgaB without the predicted signal peptide and lipidation site and fused to an \textit{N}-terminal thrombin-cleavable His\textsubscript{6} tag. Transformed cells were grown in 1L of Luria Broth (LB) media (25 g/L) with 100 µg/ml streptomycin at 37 °C. When the culture reached mid-log phase (OD 0.5-0.6), protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. The cells were then incubated while shaking overnight at 10 °C. Cells were harvested by centrifugation for 1 hour at 3452 xg and each liter was resuspended in 10 ml lysis buffer (50 mM phosphate, 300 mM NaCl, pH 8), followed by the addition of 1 mini protease inhibitor mixture tablet (Roche Applied Science). The cells were then lysed by sonication; 10 seconds intervals of on and off for 5 minutes, then cell debris was removed by centrifugation for 45 min at 16260 xg. The resulting supernatant was incubated with 1 ml of Ni-NTA Agarose resin (QIAGEN), which was pre-equilibrated with lysis buffer, for 45 minutes at 4°C. The mixture was spun for 5 minutes at 3452 xg to remove the supernatant. The resin was put in a column (poly-prep chromatography columns 10 ml, BioRad) washed with 10 column volumes of lysis buffer and eluted with 5-100 mM imidazole gradient (each fraction is 1 ml; 4 fractions of 5 mM, 3 of 10 mM, 20 mM, 50 mM, and 100 mM). Fractions were analyzed by SDS-PAGE (Fig. 18). The gel shows that there are some impurities of molecular weight less than 50 kD so the fractions containing His-PgaB were concentrated using an ultra-0.5 centrifugal filter device (Amicon Ultra 50K device, Millipore) to result in pure PgaB protein. Purifications yielded 12-15 mg of purified recombinant protein per 1L of culture. The protein solution was quantified by nanodrop using a molar extinction coefficient of 15200 M\textsuperscript{-1}cm\textsuperscript{-1}. Protein was stable at 4 °C for 2 weeks without significant loss of activity.
4.3 Enzymatic reactions

4.3.1 Polymerization Reaction

The procedure described by Kawase\textsuperscript{58} was followed. Membrane extracts of PgaCD (1 mg/ml-20 mg/ml)) were incubated at 37 °C with UDP-GlcNAc (10-20 mM), c-di-GMP (20 µM), D-cycloserine (100 µM), and alkaline phosphatase (0.02 units/µL) in HEPES buffer (50 mM HEPES, 5 mM CaCl\textsubscript{2}, 5 mM MgCl\textsubscript{2}, pH 7.0). Concentration of PgaCD was calculated using BCA assay. D-cycloserine was added to inhibit peptidoglycan biosynthesis and c-di-GMP was added to activate the PgaCD synthase and promote binding of PgaC and PgaD. Alkaline phosphatase was added to break down UDP, the by-product from the polymerization reaction, to prevent it from inhibiting the PgaCD complex.

4.4 Direct Analysis in Real Time Mass Spectrometer (DART-MS)

A JEOL AccuTOF model JMS-T1000LC mass spectrometer equipped with a Direct Analysis in Real Time (DART) ion source was used for all the DART studies.
The following conditions all had to be optimized in order to get the best signal possible for the acetate in solution;

1. Temperature of the He: 200 °C.
   Temperatures investigated: 150, 200, 250, 300, 350, 400, 500 °C
2. Volume of sample: 2 µL
   Volumes investigated: 1, 2, 3, 5 µL
3. Voltage of the quadruples: 500 V
   Voltages investigated: 300, 400, 450, 500, 550, 600, 650 V
4. Distance between the dart source and the mass spectrometer inlet: 5 cm
   Distances investigated: 2, 3.7, 5 cm
5. Whether to use the interface or not: better signal was observed without the use of interface
6. Speed of the rail: 0.2 mm/sec
   Speeds investigated: 0.2, 0.3, 0.5, 1, 3 mm/sec
7. Time sample was allowed to dry on the capillary tube before analyzing it: 10 minutes
   Time periods investigated: 0, 1, 2, 3, 5, 8, 9, 10 minutes
8. Concentration of propionate (internal standard): for reactions with 20 mM sugar 0.5 mM propionate was used.

Melting point capillary tubes (1.5-1.8 x 90 mm) on capillary tube holder were washed with deionized water, then a heat gun was used to dry the tubes. 2 µL of sample was pipetted on each capillary tube. The samples are allowed to dry for 10 minutes at ambient conditions before the capillary tube holder is loaded on the DART rail. (Fig. 19)
Each sample is loaded on all the capillary tubes, so each capillary tube is a technical replicate (10 tubes on the capillary tube holder which means that there are 10 replicates for each sample). After the run a m/z spectrum is produced, then extracted ion chromatograms for masses 59 and 73 are produced. Extracted ion chromatogram (EIC) for m/z 59 shows the signal for this specific mass in each sample (each peak in fig. 20 b and 20c represent one capillary tube). The ratio between the intensity of the peak in 59 EIC and that corresponding to the peak in 73 EIC is taken and used for analysis.

Fig 19: It shows the set up for the DART-MS; the capillary tube holder on the rail in between the DART source and mass spectrometry inlet, in the reaction zone.

Fig 20: a) m/z spectrum for one of the samples, b) Extracted ion chromatogram for acetate (m/z = 59), c) Extracted ion chromatogram for propionate (m/z = 73).
4.5 Fluorescamine assay

The de-N-acetylation reaction of PNAG oligomers by PgaB was monitored using fluorescamine. This dye reacts with primary amines in solution to produce a fluorescent product ($\lambda_{\text{ex}} = 360$ nm, $\lambda_{\text{em}} = 480$ nm). To 10 µL aliquot of deacetylation reaction mixture, 20 µL of 0.5 M borate buffer (pH 9) and 10 µL of freshly prepared 20 mg/ml fluorescamine solution in dimethylformamide (DMF) were added and mixed by pipetting. After 10 minutes at room temperature, 80 µL of water was added. An 80 µL aliquot of solution was removed from each sample and transferred to a Corning 3686 half-area 96-well plate for measurement in a CLARIOstar plate reader (BMG LABTECH), 360-nm excitation, 460 nm emission, 5 nm slit widths. Glucosamine solutions were used as standards to calculate amine concentration.
5 Bibliography


22. Little, D. J. *et al.* The structure- and metal-dependent activity of Escherichia coli PgaB provides insight into the partial de-N-acetylation of poly-β-1,6-N-acetyl-D-glucosamine.


52. Rummel, J. L., McKenna, A. M., Marshall, A. G., Eyler, J. R. & Powell, D. H. The coupling of direct analysis in real time ionization to fourier transform ion cyclotron


