BACTERIAL GLYCAN IMMOBILIZATION FOR INTERACTING PARTNER ANALYSIS

by

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ABSTRACT

ALEXIS HOPE MURRAY. Bacterial glycan immobilization for interacting partner analysis. (Under the direction of DR. JERRY TROUTMAN)

Bacterial cell surface glycans mediate pathogenic and symbiotic interactions with hosts and other organisms. The implications of bacterial glycans in human health have made them attractive targets in drug design for antibiotics, antibacterial vaccines, and even in new therapeutics for treating diseases. Despite this appeal, there are a lack of specific glycan-interacting tools to isolate, detect, and target bacterial glycans. This is partly due to the incredible diversity of bacterial glycans, which includes rare monosaccharide building blocks as well as complex branching and linkages. However, many bacterial polysaccharides are composed of oligosaccharide repeat units, and these repeat units are built on a common isoprenoid scaffold called bactoprenyl phosphate (BP). While we have used this knowledge for a generalized route for studying bacterial glycans, there is a need for more efficient ways to isolate glycans such as polysaccharides and a need for methods to develop glycan-interacting tools for a diverse set of bacterial glycans.

The work in this thesis followed a general scheme for developing new bacterial glycan-interacting partners (GIPs), which included: glycan isolation and glycan immobilization onto a magnetic bead (MB) platform for GIP analysis. This study utilized a model bacterial glycan system with the well-studied capsular polysaccharide A (CPSA) from *Bacteroides fragilis* and the isoprenoid-linked tetrasaccharide CPSA repeat unit. Using these two glycans, this study investigated two glycan immobilization strategies, which may be applied to other bacterial glycans. A new isolation technique and noncovalent immobilization method were established for the isoprenoid-linked oligosaccharide, and a preliminary covalent immobilization method was investigated for the polysaccharide.

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DEDICATION

To my parents, for your confidence and support.

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LIST OF ABBREVIATIONS

2AB	2-anthranilamide
AMAC	2-aminoacridone
AP	alkaline phosphatase
B. fragilis	*Bacteroides fragilis*
BP	bactoprenyl phosphate
BPP	bactoprenyl diphosphate
C. jejuni	*Campylobacter jejuni*
2CN-BP	2-cyanoanilino bactoprenyl phosphate
CPS	capsular polysaccharide
CPSA	capsular polysaccharide A
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
E. coli	*Escherichia coli*
ECA	enterobacterial common antigen
FITC	fluorescein isothiocyanate
FLD	fluorescence detector
FPLC	fast protein liquid chromatography
GBP	glycan-binding protein
GIP	glycan-interacting partner
Glc	glucose
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography

IEF	isoelectric focusing
IEX	ion exchange
kDa	kiloDalton
LB	lysogeny broth
LC-MS	liquid chromatography mass spectrometry
LOS	lipooligosaccharide
LPS	lipopolysaccharide
m/z	mass/charge ratio
MB	magnetic bead
MF	membrane fraction
MWCO	molecular weight cutoff
Ni-NTA	nickel nitrilotriacetic acid
NMR	nuclear magnetic resonance
OD_{600}	optical density at 600 nm
PBS-T	phosphate buffered saline with Tween
PCR	polymerase chain reaction
PglB	a glycosyltransferase
PglF	a dehydratase
PGT	a phophoglycosyltransferase
GT	glycosyltransferase
SDS-PAGE	sodium dodecyl-sulfate polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
SPE	solid phase extraction

SIM	selected ion monitoring
ssDNA	single stranded deoxyribonucleic acid
UDP-AATGal	uridine diphosphate-linked N-acetyl-4-aminogalactosamine
UDP-GalNAc	uridine diphosphate-linked N-acetylgalactosamine
UDP-GlcNAc	uridine diphosphate-linked N-acetylglucosamine
V. vulnificus	*Vibrio vulnificus*
WaaL	a ligase
WAX	weak anion exchange
WcfM	a mutase
WcfN	a glycosyltransferase
WcfO	a pyruvyltransferase
WcfP	a glycosyltransferase
WcfQ	a glycosyltransferase
WcfR	an aminotransferase
WcfS	a phosphoglycosyltransferase

CHAPTER 1: INTRODUCTION

1.1: Bacteria in Human Health

There are many connections between bacteria and human health, which researchers have studied to treat bacterial infections and design new therapeutics. While antibiotics are widely used to treat bacterial infections, there are also established health benefits associated with probiotics, which have been shown to help a variety of aspects of human health including immune health. However, there are risks with probiotics including interference with drug efficacy and toxicity, invasive infections from translocated organisms, antibiotic resistance transfer, and there are a lack of long-term safety studies.[1] Further, the problem of antibiotic resistance in pathogenic organisms is pressing, and there is a need for new drugs to treat infections.

Combinatorial chemistry can be used to synthesize large, variable libraries of synthetic drug candidates, and high-throughput screening technology has enabled the rapid selection of bacterial-interacting molecules. However, the mechanism of interaction is often unknown or nonspecific to a bacterial species. Further, broad-spectrum antibiotics kill many bacteria in the environment, which exacerbates antibiotic resistance prevalence by enrichment of resistant organisms in the natural gene pool. While there are many available antibiotics, pathogenic bacteria are adapting rapidly. A preventative approach for treating bacterial infections include whole-cell vaccines such as the early diphtheria-tetanus-whole-cell pertussis (DTwP) vaccine, which inoculates the patient with killed bacteria. However, these vaccines can have adverse side effects, which has led to the development of acellular antibacterial vaccines.

To aid in both antibacterial vaccine development and improved antibiotic design, understanding the structure of biomolecules can aid in the rational design of antibacterial agents which specifically target certain pathogenic organisms. For instance, elucidation of specific antigens, such as the pertussis toxin, on bacterial cell surfaces have enabled the development of acellular subunit vaccines. Further, understanding antibacterial mechanisms of action helps improve the design of new antibiotics. For instance, Stokes and coworkers elucidated the mechanism of action of vancomycin and showed structural changes in the outer membrane composition of both resistant and vancomycin-sensitive bacteria.[2] Studies on bacterial surface biomolecules can reveal specific antibiotic-bacterial interactions and provide general insights into improving antibiotic design.

This rational drug design approach is also applicable to the development of new therapeutic agents for treating diseases. As clinical strategies for treating bacterial infections have shifted away from whole cell vaccines, drugs derived from symbiotic bacteria will likely move away from live probiotics as more research is established on probiotic mechanisms and new therapeutics are developed. For instance, understanding the mechanism by which symbiont factors regulate symbiotic interactions with hosts can help researchers design more effective drugs and may avoid adverse risks associated with live microorganism treatments. As an example, probiotics are not recommended for immune compromised individual who may potentially benefit from the immunomodulatory effects associated with probiotics. Studying the mechanisms for how probiotic organisms improve immune health is increasingly important as there is a rise in the cases of immune mediated inflammatory diseases (IMIDs), which currently do not have any cures.

1.2: Bacterial Glycans

The molecules on bacterial cell surfaces are at the interface of many complex interactions with hosts and other organisms. Studying these biomolecules helps elucidate new virulence and symbiont factors, which mediate pathogenic and symbiotic interactions. Elucidation of the molecular basis of these interactions helps in rational drug design for treating bacterial infections and in finding new therapeutics for diseases. Bacterial cell surfaces are covered in a layer of glycans called the glycocalyx, and there a wide variety of complex glycans, some of which distinguish bacterial species and subspecies serotypes (Figure 1).

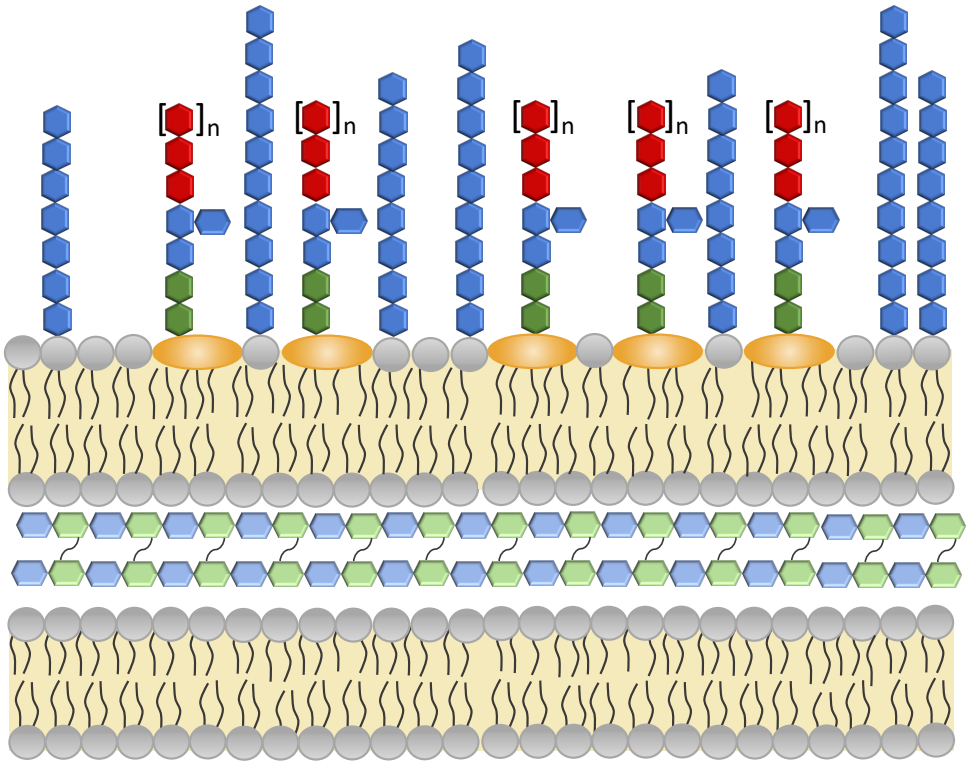

Figure 1: Gram negative bacterial cell surface covered in glycans (hexagons) with a lipid bilayer and thin layer of peptidoglycan.

Mammalian glycosylation in many aspects of human health and disease are more well-known than the bacterial glycome, and this gap in knowledge is partly due to bacterial glycan

diversity. Currently known bacterial monosaccharides have a ten-fold greater diversity than the monosaccharide building blocks in mammalian systems.[3] This monosaccharide diversity is further complicated by the variety of glycan linkages and branching combinations in complex bacterial glycans.

While there are many types of bacterial glycans, I will mostly focus on specific types of bacterial surface glycans from Gram negative bacteria including lipopolysaccharides (LPS), lipooligosaccharides (LOS), and capsular polysaccharides (CPS). While many bacterial polysaccharides are composed of rare monosaccharides and can have complex branching, they are often composed of repeat unit oligomers, which simplifies structural analysis. Additionally, these repeat oligomeric units are commonly assembled on a C_{55} isoprenoid called bactoprenyl phosphate (BP). General glycan assembly is initiated by the appendage of a phosphosugar from nucleotide-linked sugar donors onto the BP anchor, which is catalyzed by a phosphoglycosyl transferase (PGT) enzyme (Figure 2). This step is generally followed by the sequential addition of monosaccharides from a variety of nucleotide-linked sugars by glycosyltransferases (GTs).

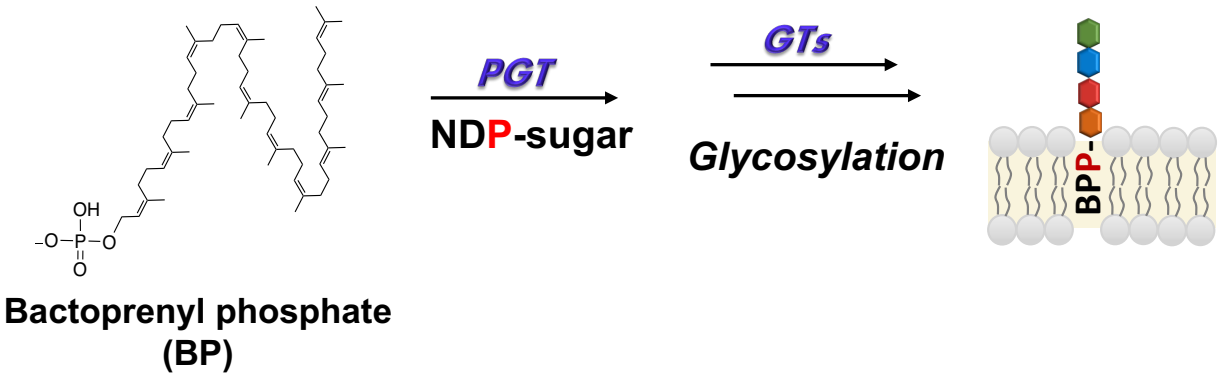

Figure 2: General bacterial BP-linked oligosaccharide assembly pathway scheme.

Knowledge of this common pathway and the oligomeric repeat units that make up bacterial surface polysaccharides are important advantages for developing new tools to study bacterial glycans and their biosynthesis pathways. There is still significant work for characterizing the function of specific PGT and GT enzymes and in the development of tools for probing their specificity. Further, there is a lack of glycan-interacting tools to isolate, detect, and target specific bacterial glycans for fundamental research and biomedical applications.

1.3: Methods for Bacterial Glycan Isolations

1.3.1: Chemoenzymatic Preparation of Bacterial Glycans

The isolation and analysis of biomolecules such as protein and nucleic acids have been expedited by modern sequencing technologies such as next generation sequencing (NGS). Sequence analysis applied in high-throughput analytical platforms has helped build global maps of complex biological systems.[4] From genomic data, researchers can analyze local genes involved in a predicted biochemical pathway, and the function of each protein-encoding gene can be studied using molecular biology techniques. In contrast to proteins and nucleic acids, glycan production is not template-encoded, which eliminates the direct application of sequencing for studying glycans. Alternatively, the proteins in predicted glycan biosynthesis pathways can often be isolated and used in chemoenzymatic reactions to study glycan production.

A predicted biosynthetic pathway can be identified by analyzing genetic data from a target organism, and the sequencing data can be referenced against library sources of biomolecular information. These comparisons can be used to identify sequence homology, which is used to predict the function of an unknown gene or gene cluster.[5] While sequence analysis is

helpful for identifying potential glycan biosynthetic pathways, the function of each translated protein in the predicted pathway must be demonstrated. One way to study enzymatic function is by recombinant expression and isolation of the protein from *E. coli* or other model organisms. The target gene sequence is inserted into a vector and transformed in a chosen cell line for protein expression. Affinity purification tags such as histidine residues are often genetically installed at the N- or C-terminus of target protein sequences for rapid purification applications like immobilized metal affinity chromatography (IMAC).[6] The information from sequence or structural data of the gene or protein can be used to design experiments and study the isolated protein activity.

Proteins that are difficult to isolate in a functional form can be substituted for homologous enzymes from non-native pathways. For instance, the Troutman group substituted the *B. fragilis* native dehydratase UngD2 for the previously studied *C. jejuni* dehydratase PglF and successfully utilized the heterologous system to study downstream enzymatic activity.[7] Once the functions of the proteins are demonstrated, the chemoenzymatic reaction conditions can be scaled up to obtain milligram up to gram-scale quantities of the target glycan.

While chemoenzymatic glycan production strategies offer tunable *in vitro* conditions to study glycosylation and enzyme function, optimization can be time consuming. Further, the preparative scalability of these glycan production methods is often limited by expensive substrates. These substrates include costly nucleotide-linked sugar precursors and enzyme cofactors such as acetyl coenzyme A ($65.70/mg from Sigma). In some cases, nucleotide-linked sugars, such as the *E. coli* enterobacterial common antigen (ECA) acetyltransferase WecD

substrate, dTDP-Fuc4NH_2, are not commercially available. To circumvent these limitations, additional chemoenzymatic assembly can be used to synthesize glycan assembly precursors before building the target glycan.[8, 9]

Chemoenzymatic glycan assembly provides access to a library of nucleotide sugar donors, but the product yield is ultimately limited by enzymatic turnover. For instance, UDP-GalNAc is a common nucleotide-linked sugar in many bacterial glycan assembly pathways but is expensive to purchase commercially ($3,740 for 100 mg from Sigma). The Troutman lab was able to limit the cost of UDP-GalNAc with the N-acetyl hexosamine 4-epimerase WbpP from *V. vulnificus* to convert UDP-GlcNAc ($293 for 100 mg from Sigma) to UDP-GalNAc. While this system was advantageous for cutting the cost of bacterial glycan assembly, the conversion ratio of UDP-GlcNAc to UDP-GalNAc is poor (7:3), and it can be difficult to separate these isomers.[10] One strategy to overcome poor enzymatic conversion includes multi-enzyme cascades, which can be used to couple nucleotide sugar donor production pathways, substrate recycling or regeneration pathways, and complementary glycosyltransferase (GT) reactions. For example, Shao and coworkers developed a six-enzyme UDP-GalNAc regeneration system, which was coupled directly to oligosaccharide assembly reactions as a cost effective strategy for synthesizing GalNAc-containing glycans.[11]

The flexibility in coupling chemoenzymatic reactions together is partly due to the high regio- and stereoselectivity of many GTs and other sugar modifying enzymes. This enables enzymatic reactions to be coupled in one-pot synthesis reactions without intermediate purification steps, which improves the overall yield. Notably, the selectivity of enzymes often

simplifies chemoenzymatic synthesis when compared to purely synthetic strategies, which can require multistep protection-deprotection reactions and subsequent purification to accomplish selective glycan modifications.[12]

While chemoenzymatic and synthetic methods for bacterial glycan production are viable options for obtaining mono or oligosaccharides, these methods are relatively limited for bacterial polysaccharides. Polysaccharide preparation methods are also crucial because the glycan length can significantly impact the biochemical properties of the glycan. For instance, both the charged monosaccharide components and the number of oligomeric units in zwitterionic polysaccharides, such as the *Streptococcus pneumoniae* serotype 1 (Sp1) capsular polysaccharide, are important for their unique immunomodulatory properties.[13, 14] Current challenges for synthetic production of bacterial polysaccharides include lengthy multistep synthesis and the preservation of charged, labile groups during preparation.[15] In the case of chemoenzymatic glycan synthesis, polymerase enzymes required for many bacterial polysaccharide pathways are difficult to utilize *in vitro*. However, there is promise in combining chemoenzymatic and modern synthetic glycan production strategies for polysaccharide assembly.

The time cost associated with synthetic carbohydrate production methods has been minimized with advancements in automated synthesis. For instance, solid phase automated glycan assembly (AGA) is used to assemble glycans on a solid support and washing steps replace tedious purification steps. However solid-phase methods often use excess expensive glycosyl donors, can have low yields due to poor glycosidic bond formation on solid supports, and glycans are often limited in size.[16,17] Recently, Ye and coworkers developed an automated

solution-phase glycan synthesizer, which used stoichiometric amounts of glycosyl donors and synthesized a record 1080-mer polysaccharide with 4320 stereogenic centers.[18, 19] This accomplishment greatly surpassed previous limitations of solid-phase methods for glycan synthesis and offers promise to produce polysaccharides on a multigram-scale. Notably, these methods are still limited for prokaryotic glycans, and significant work is needed to extend these strategies to complex bacterial polysaccharides.

Coupling chemoenzymatic gram-scale production strategies for obtaining libraries of glycosyl donor building blocks and recent advancements in automated polysaccharide synthesis offers a promising strategy for polysaccharide production. In the case of bacterial polysaccharides, future work still needs to be done to test the applicability of automated polysaccharide assembly for different types of glycan linkages and rare bacterial monosaccharides including charged sugars, which are often important for the immunogenic properties of polysaccharides.[14, 17]

1.3.2: Isolating Glycans from Bacteria

Well established methods for obtaining bacterial polysaccharides include isolation from the native organism or by isolation of the recombinant material in a model organism. There are a wide variety of methods for isolating different types of bacterial glycans from cells, which can vary based on the chemical composition and biological location of the target glycans.[20, 21] While there are many available purification methods, isolation of polysaccharides from many native organisms is often fundamentally inefficient. For instance, substrates required for glycan assembly can be sequestered by other endogenous glycan assembly pathways, and in some cases

the recombinant glycan can be incorporated into nontarget pathways and produce alternate glycoforms.[22-24] These biological processes can diminish the product yield and complicate isolation.

Innovations in the fields of metabolic and genetic engineering have enabled researchers to construct more efficient biological cell factories by genetic manipulation. One of the first marketed examples of this is the recombinant production of human insulin by engineered strains of *E. coli*.[25] While microbes have long been used as drug factories, it is becoming apparent that bacterial cell surfaces are also inherently rich sources of information for rational drug design and therapeutics. The study and isolation of polysaccharides has been crucial for development of antibacterial glycoconjugate vaccines such as Prevnar-20, which protects against 20 types of pathogenic pneumococcal bacteria.[26] In addition to the specific polysaccharide of each bacterial serotype (20 polysaccharides in the case of Prevnar-20), another key component of these vaccines is the immunogenic carrier, which is covalently conjugated to one or multiple types of polysaccharide antigens. The carrier is crucial for most carbohydrate-based vaccines because, unlike other antigens such as lipids and proteins, most carbohydrates do not elicit a T cell-dependent immune response, which does not produce the desired memory immune response to the pathogen and diminishes vaccine efficacy.[27, 28] While a carefully chosen carrier can improve glycoconjugate vaccine efficacy, the development of new vaccines is vastly limited by time-consuming polysaccharide isolation and synthetic glycan-carrier conjugation optimization.

As an alternative to synthetic glycan-carrier methods, bioconjugation strategies utilize biological systems to couple glycans and carriers for *in vivo* glycoconjugate vaccine production.

These systems avoid time-consuming polysaccharide purification, and benefit from the convenience of the *in vivo* protein glycan coupling technology (PGCT). Recently, Terra and coworkers developed an engineered strain of *E. coli* with a recombinant bioconjugation system to rapidly produce new glycoconjugate drug candidates.[29] An important factor in this *in vivo* glycoconjugate vaccine development system includes the oligosaccharyltransferase, PglB, which is a protein-glycan coupling enzyme in the *C. jejuni* N-linked heptasaccharide biosynthesis pathway.[30] In addition to studies on PglB activity, recombinant PglB and its modified forms have been used to conjugate non-native polysaccharides to a variety of protein scaffolds.[30, 31] An important addition to this *in vivo* glycoconjugate vaccine development system by Terra and coworkers is in the glycoengineered *E. coli* strain, which included deletions of select genes to maximize incorporation of the constructed glycan to the target PglB protein-conjugate pathway.[29] This glycoconjugate production system is an excellent example of using glycoengineering to improve glycan production efficiency in biological cell factories.

Once a native or recombinant bacterial glycan production system is selected, there are many different glycan isolation methods. Most Gram negative bacterial polysaccharide isolation protocols follow a sequence of bacterial lysis, liquid-liquid extraction, enzymatic digestion, alcohol precipitation, and chromatographic purification.[32] During bacterial polysaccharide purification, endotoxin removal is vital to preventing interference in upstream applications. For instance, endotoxin can cause complications such as sepsis and is intensely screened during vaccine production.[33]

Bacteria can shed endotoxin, or lipopolysaccharide (LPS), and it can be present either endogenously in the bacterial expression system or in the environment. Endotoxin has several forms including deep rough, rough, and smooth lipopolysaccharide. Smooth LPS contains a lipid A moiety covalently linked to inner and outer core oligosaccharides with a distal polymer. The rough lipooligosaccharide (LOS) includes lipid A core without a distal polymer, while the deep rough variation only contains lipid A linked to an inner core.[33] The variable chemical properties of biologically active endotoxin can make it difficult to completely remove during polysaccharide purification.

Common bacterial polysaccharide isolations generally start with a hot-phenol water extraction (45:55 v/v), which is used to lyse bacteria and separate most glycans and lipids into an aqueous and organic layer, respectively.[34] This method is commonly used for LPS extraction, where smooth LPS predominantly partitions to the aqueous layer, and other LOS forms are in the organic layer. An additional ethyl ether extraction is then used to remove residual phenol and the aqueous layer is exhaustively dialyzed before enzymatic digestion. While this method can have high yields for polysaccharide extractions, it does not effectively separate polysaccharides from endotoxin.

In contrast to the hot-phenol water method, a single and two-phase Bligh-Dyer (1:2:0.8 and 2:2:1.8 v/v/v chloroform/methanol/water) solvent system enables bacterial lysis and the separation of polysaccharides from most endogenous endotoxin.[35] Most smooth LPS is insoluble in the single-phase solution and can be pelleted by centrifugation. In the two-phase solution, the organic layer with lipid A and some LOS forms can be separated from the polysaccharide-

containing aqueous layer.[33, 36] While Bligh-Dyer extractions partition most lipids and some lipid-linked oligosaccharides to the organic layer, certain lipid-linked oligosaccharides are predominantly soluble in the aqueous layer.[37] In particular, charged sugar moieties such as glucuronic acid seem to mediate the shift of lipooligosaccharides from the organic to the aqueous layer.[37]

While liquid-liquid extractions coupled with enzymatic digestion can eliminate many biological contaminants such as proteins and nucleic acids, additional chromatographic purification of the polysaccharide is often required. Some methods include ion exchange and gel filtration or size exclusion chromatography for further polysaccharide purification. If these methods are still ineffective for fractionating endotoxin from other biomolecules, there may be interactions between endotoxin and proteins, nucleic acids, or polysaccharides in the sample. Before and during chromatographic separations, metal chelators and detergent additives can be used to minimize endotoxin aggregation.[33]

While the bacterial glycan isolation process can be lengthy, polysaccharide production efficiency has been improved through recombinant expression and glycoengineering. Polysaccharide isolation is often an essential step precluding *in vitro* study of bacterial polysaccharides. The challenge is to develop more efficient glycan purification methods, which preserve the native glycan structure and to apply analytical methods to critically evaluate these isolated materials.

1.4: Tools for Glycan Analysis

1.4.1: Methods for Glycan Analysis

Both fast protein liquid chromatography (FPLC) and high-performance liquid chromatography (HPLC) are used for analytical and preparative separations of various biomolecules including glycans. Compared to FPLC stationary phases, HPLC packings have smaller particle diameters, which contributes to a higher column separation efficiency and causes higher back pressure.[38] Many FPLC stationary phases are made of a resin with low mechanical stability and are designed with larger particle sizes that alleviate back pressure. These types of column packings also have a wide pH stability range that can accommodate chemically rigorous column cleaning and regeneration methods. Conversely, the silica-based stationary phases in most HPLC columns can tolerate high back pressure but have a limited pH range (between 2-8) since exposed silanol groups are subject to degradation at low and high pH. Advancing from an analytical scale, preparative HPLC columns are designed for increased loading capacity over analytical columns, but they can cost up to ten times more than FPLC columns with a similar capacity.[39]

Before glycan analysis, sample cleanup is generally utilized to preserve the column and to reduce interference with the analyte. Some sample cleanup steps include filtration, liquid-liquid extraction, and solid-phase extraction methods. Other pre-analysis treatment steps such as chemical hydrolysis or enzymatic cleavage can be used to separate glycans from conjugated moieties or to digest complex glycans into oligo- or monosaccharide components. Since carbohydrates do not strongly absorb UV and visible light, detection methods such as UV-vis and fluorescence are not sufficient for routine carbohydrate analysis. However, glycans can be

labeled with a chromophore or fluorophore via pre- or post-column derivatization, which is compatible with these optical detection methods.[40]

Column stationary phases for glycan analysis include a wide variety of functionality depending on the charge and length of glycan as well as conjugated moieties. Ion exchange, size exclusion, normal phase, and hydrophilic interaction chromatography (HILIC) are some common stationary phases for glycan analysis, while reverse phase stationary phases are used for analysis of derivatized glycans, glycoproteins, and glycolipids.[41] Several of these LC separation modes can be coupled directly to mass spectrometry (MS) detection, which is convenient for native glycan analysis. For instance, targeted analysis of cell lysates can be used to demonstrate putative gene functions in a glycan biosynthesis pathway. The Troutman lab constructed sequential *E. coli* deletion mutants for the colanic acid pathway and identified accumulated colanic acid intermediates in cell lysates by LC-MS, which demonstrated individual gene functions.[37]

While LC-MS analysis is often convenient for oligosaccharide analysis, the molecular weight cutoff of most LC-MS instruments limits the application for polysaccharide analysis. In the field of proteomics and genomics, large biomolecules can be enzymatically digested into discrete fragments, akin to molecular fingerprints, and this information can be analyzed against databases in a high-throughput format.[42] Due to the structural heterogeneity of polysaccharides, there are a lack of broadly applicable reagents for reproducible and structurally informative polysaccharide cleavage. Acid hydrolysis can be used to digest polysaccharides into oligosaccharides for analysis, but these methods require optimization for different samples.[43]

Other polysaccharide characterization techniques include NMR analysis, which is powerful tool for identifying pure polysaccharide samples. However, this technique requires a significant amount of material and is not convenient for routine analysis of naturally abundant glycans in biological systems. A huge breakthrough in high-throughput glycomics was in the development of glycan microarrays, which have enabled the rapid detection and quantification of many samples simultaneously.[44, 45] Current challenges in expanding the application of glycan microarrays is in the development of specific glycan detection reagents to span the diverse pool of bacterial glycans.

1.4.2: Glycan Analysis Reagents and Platforms

Glycan array development usually requires the preparation and immobilization of glycans before glycan-binding analysis. Glycan preparation typically includes the isolation and characterization of a target glycan, usually from naturally derived sources, which can be tedious. An alternative approach includes shotgun glycomics, which enables the analysis of many undefined glycans from biological samples and selects targets against an antigen of interest before glycan characterization.[46] Glycan microarrays have helped elucidate a variety of glycan-protein interactions in nature, and there has been considerable work done in characterizing the interactions between glycans and glycan-binding proteins (GBPs).

In addition to elucidating fundamental biological interactions, glycan-interacting partners (GIPs) are intriguing and dynamic investigation tools. For example, these probes simplify analysis and isolation techniques and can be used in affinity chromatography, cell surface

imaging, and in clinical and diagnostic applications. A downside to this approach is that the known GIPs do not cover the vast diversity of glycans, and some of these interactions are weak or nonspecific. For instance, there are numerous colorimetric and fluorescent assays that are used to quantify carbohydrates in a sample.[47, 48] Some of these assays use nonspecific chemical reagents to quantify the total carbohydrate content, while others use reagents that specifically interact with a particular glycan. For instance, Sadler and coworkers measured both the specific colanic acid (CA) content in the bacterial cell growth medium and the total carbohydrate content of the EPS to optimize CA production in engineered *E. coli*.[49] Another widely applicable specific polysaccharide detection method utilizes glycan-interacting biomolecules such as GBPs to detect glycans.

Interestingly, most carbohydrate-protein interactions in nature are relatively weak (μM affinity).[50, 51] Many GBPs achieve high binding avidity through multivalent interactions with glycans, and this binding effect, termed the cluster glycoside effect, is greater than the sum of monovalent interactions.[50] An example of this effect are GBPs interacting with densely coated glycans on a cell surface or through interactions with multiple repeat units on a polysaccharide. These multivalent carbohydrate-protein interactions are considered and used by researchers in applications such as glycoconjugate vaccine design, lectin isolation, and in the selection of GBPs through multivalent scaffolds and screening platform displays.[52, 53] For example, Imperiali and coworkers utilized a multivalent GBP yeast display platform and obtained an engineered lectin for the Thomson-Friedenreich antigen (μM affinity) from a library of 1.4×10^9 proteins.[53]

Despite efforts to engineer better lectins (<uM Kd), there are few examples of lectins with both a strong affinity and selectivity for glycans.[5] Antibodies are also common GBPs used

for glycan detection, and isolated antibodies can have high selectivity for a target glycan. However, antibody production requires animals, and the development of highly specific monoclonal antibodies is expensive. Further, due to the poor immunogenicity of most glycans, these antibody preparations can require large amounts of the glycan to produce glycan-specific antibodies.[54]

While GBPs are valuable glycan-interacting tools, there are also other types of important GIPs. DNA-encoded or other nucleic acid-based libraries benefit directly from sequencing technology because the sequences act as molecular barcodes for library members. These sequences can be identified and amplified by polymerase chain reaction (PCR). Further, this strategy enables other researchers to use these sequences directly and rapidly obtain GIPs without the need for protein purification or other processes for obtaining GBPs. As an alternative to GBPs, aptamers are chemically synthesized single-stranded RNA or DNA (ssRNA/DNA) molecules with binding affinities for a wide variety of targets that are competitive to antibodies. Compared to antibodies, ssDNA aptamers are more stable, have potential for well-defined modifications, and are inexpensive and faster to produce ($50/mg versus $2,000-5,000/mg of antibodies).[55] Synthetic aptamer libraries are typically on the scale of up to 10^{15} candidates, and there are many examples of aptamers that bind to small molecules, whole cells, and biomolecules such as cell surface proteins and glycans. For instance, aptamers have been developed against bacterial whole cells for various pathogens including bacteriostatic aptamers against *Salmonela*.[56, 57] An alternative strategy includes aptamer screening against bacterial molecular recognition elements (MREs) such as protein-based pathogenic toxins and some glycans such as peptidoglycan and lipopolysaccharide (nM affinity).[57-59]

Following GIP library production and target glycan isolation, another fundamental step includes glycan immobilization onto a platform. High-throughput, one-pot library screening enables the selection of GIPs, where time associated with the experiment is independent of the library size.[60] Many glycans can be immobilized on slides or multivalent scaffolds either by covalent modifications or by noncovalent interactions. Noncovalent immobilization proceeds via adsorption of the glycan or conjugated moiety. For instance, polysaccharides have been immobilized on nitrocellulose-coated glass slides and other surfaces for analysis of interactions with monoclonal and polyclonal antibodies.[45, 61] These methods have been used to rapidly analyze microbial polysaccharides with a small amount of sample, but are limited in application for low molecular weight oligosaccharides.[62] Noncovalent immobilization methods can also have problems with reproducibility due to the heterogenous distribution of exposed binding sites on the surface. Alternatively, site-specific noncovalent immobilization methods capture the glycan via naturally or synthetically conjugated moieties. Tang and coworkers chemically conjugated glycans to lipids and immobilized these neoglycolipids via the lipid handle onto microtiter plates for probing glycan-protein interactions.[44] These noncovalent immobilization methods often require careful tuning of washing conditions to avoid dissociation of the noncovalent interaction during analysis.

Covalent glycan immobilization can be achieved directly via the capture of the free glycan reducing end or by moieties on pre-derivatized glycans. For instance, Park and coworkers immobilized unmodified glycans onto hydrazine-coated glass slides.[63] Other methods immobilize pre-derivatized glycans on epoxy or N-hydroxysuccinimide (NHS)-coated surfaces

via the conjugated amine or by oxime or hydrazone formation.[64, 65] These methods are notably only applicable for reducing glycans, and imine or Schiff base formation during modification changes the native reducing end glycan structure, which is important for glycan binding.[66] Recently, the Troutman lab chemoenzymatically built the *C. jejuni* heptasaccharide onto an azide-linked isoprenoid analogue via click chemistry onto dibenzocyclooctyne (DBCO) magnetic beads.[10] This system requires knowledge of the specific glycan biosynthesis pathway but mimics the natural presentation of isoprenoid-linked bacterial sugars and benefits from the improved specificity of the click-chemistry handle.

While covalent immobilization methods are generally preferred over noncovalent methods, covalent modifications modify the native glycan structure. There are few site-specific and modification-free methods for probing glycans in a high-throughput format. Further, there is a great need for generally applicable methods for the isolation of a variety of different types of glycans. Notably, there are distinct advantages in using magnetic beads (MBs) to immobilize glycans because glycan-binding partners can be easily selected on the MBs with commercially available magnetic decanters, which are available in high-throughput well plate formats, and the MB multivalent platform mimics glycan cell surface presentation. In this thesis I will propose a general isolation strategy for native bacterial isoprenoid-linked oligosaccharides and aim to demonstrate noncovalent and covalent immobilization strategies, which cover a variety of glycans for glycan-binding partner analysis on magnetic beads.

1.5 A Model System: Capsular Polysaccharide A (CPSA) from Bacteroides fragilis

In this section I will discuss a well-characterized bacterial glycan system, which will be used to demonstrate versatile bacterial glycan immobilization strategies in this thesis. I will also propose the direct impact of work in this thesis to research of this glycan. The model glycan discussed is capsular polysaccharide A (CPSA) – also termed PSA 1, from the human gut symbiont *Bacteroides fragilis*.[7, 67, 68] This is a suitable model because the biochemical function of enzymes in the CPSA repeat unit pathway are established, the repeat unit structure is known, and its immunological function has been investigated. CPSA is a zwitterionic polysaccharide (ZPS) with unique immunomodulatory properties and offers promise as a therapeutic for treating immune-mediated inflammatory diseases. The cumulative research on CPSA and other model ZPSs has demonstrated their potential in the development of antibacterial and cancer vaccines. However, there is still a gap in knowledge in how ZPSs specifically interact with binding partners and stimulate these immune responses. Despite the extensive research on CPSA, there is still a need for rapid CPSA capture to improve its accessibility to researchers and to improve production scalability for clinical and industrial applications.

1.5.1: Biochemical Properties of CPSA

CPSA has been isolated from the capsule of *B. fragilis* and has unique immunomodulatory properties associated with the oppositely charged amino and carboxyl groups on each tetrasaccharide repeat unit at neutral pH.[69-71] Unlike other polysaccharides which are weakly immunogenic, zwitterionic polysaccharides ZPSs, such as CPSA, can directly activate CD4+ T cells, which leads to the production of memory B cells.[32, 70, 72] The

immunomodulatory properties of CPSA shows promise in animal models to protect against inflammatory diseases such as multiple scelorosis.[73]

Another important application for ZPSs such as CPSA includes advancements in antibacterial and anti-cancer vaccine development. Like antibacterial vaccines based on pathogenic cell surface glycans, tumor-associated carbohydrate antigen (TACA)-based vaccines display antigenic carbohydrate oligomers that are overexpressed on many types of tumor cells. However, these TACA oligomers are weakly immunogenic, and immunogenic carriers can be conjugated to the target carbohydrate to induce T cell-mediated recognition of the conjugated carbohydrate epitope and boost vaccine efficacy.[74] However, interactions with the carrier also stimulates the production of carrier-specific antibodies, which can inhibit the desired immune response to conjugated antigens and even reduce the efficacy of other vaccines that utilize the same carrier.[75, 76] Therefore there is a need for new types of glycoconjugate vaccines.

While ZPSs such as CPSA are promising therapeutics, they are also important for the development of carrier-free carbohydrate-based vaccines. In some cases, adjuvants are used to enhance the immune response to the vaccine, however there are not many effective and nontoxic adjuvants.[77] Alternatively, zwitterionic polysaccharides such as CPSA have been investigated as natural adjuvants and alternative immunogenic carriers in vaccines. Andreana and coworkers have demonstrated several entirely carbohydrate-based anti-cancer vaccines using ZPSs such as CPSA to improve specific immune responses to several TACAs.[78-80] In contrast to the high antibody responses to carriers such as proteins and virus-like particles, the number of antibodies against the CPSA backbone were moderate compared to TACA-specific antibodies.[78] While the

assays in these works demonstrates the promise of CPSA as a conjugate to improve the production of specific anti-TACA antibodies in mice, there have not been studies in mouse tumor models, which are an important next step in advancing anti-cancer vaccines to clinical trials.[81]

1.5.2: A Recombinant CPSA Production System – Troutman Lab

Notably, many immunological studies on ZPSs from *B. fragilis*, including the work from the Andreana group on anti-cancer vaccines, start with a tedious polysaccharide isolation protocol.[71] These studies rely on isolation from the native organism, which requires anaerobic culturing conditions. *B. fragilis* also produces eight other capsular polysaccharides, which sequester valuable materials from the target CPSA pathway and complicate isolation. This process is time-consuming and a rapid method for isolating this valuable material will expedite future studies for investigating the therapeutic potential of CPSA.

Alternatively to native isolation, Pathan and coworkers produced the structurally defined repeat unit of the CPSA polymer in 30 synthetic steps, but this synthetic strategy was not amenable to oligomeric elongation of the repeat unit.[82] To remedy this, Wang and coworkers developed a method to generate larger CPSA fragments with up to three repeat units and a clickable handle for microarray binding partner studies.[83] While this advancement finally enabled structural studies of the CPSA oligomer, at least 7-22 repeat units are required to stimulate a T cell-mediated immune response.[84]

By chemoenzymatic methods, the Troutman lab has previously demonstrated the function of each enzyme in the construction of the isoprenoid-linked tetrasaccharide repeat unit.[68] This

characterization allowed Troutman and coworkers to construct engineered strains of *E. coli*, which produced each of the sequential repeat unit intermediates and the CPSA polymer. The work in this thesis will use these robust *E. coli* cell factories and chemoenzymatic methods to isolate the isoprenoid linked CPSA repeat unit for immobilization. Alternatively, the recombinant CPSA polymer was isolated and explored for immobilization. These immobilization platforms will help develop a glycan-interacting tools for studying CPSA. Using CPSA as a model system, I proposed to develop methods for the rapid isolation and immobilization of bacterial glycans for the high-throughput selection of glycan-interacting partners.

CHAPTER 2: MATERIALS AND METHODS

2.1: Isolation of the CPSA Repeat Unit from *E. coli*

Each sequential gene in the *B. fragilis* CPSA gene locus was expressed by Beth Scarbrough in the *E. coli* DH5α or MG1655 cell line. Starter cultures with lysogeny broth (LB) and carbenicillin (100 μg/mL) were inoculated with single colony forming units selected from carbenicillin LB-agar plates. Starter cultures were grown at 37 °C with shaking overnight and diluted 1:200 in fresh LB. Cultures were grown at 37 °C with shaking until an OD_{600} of at least 0.8 before cells were induced with 10 μM IPTG and the temperature was dropped to 16 °C overnight. Cells were harvested by centrifugation at 5,000 RCF for 20 minutes at 4 °C. The cell pellets were either stored at -80 °C for up to months at a time or immediately resuspended in deionized water for lysis.

Cells were lysed by a single-phase Bligh-Dyer solution (0.8:1:2 ratio of water, chloroform, and methanol). Insoluble components were removed by centrifugation at 2,500 RCF for 20 minutes at 20 °C. The pooled supernatants were added to a glass culture tube or separatory funnel, and a two-phase extraction was induced by the addition of water and chloroform to a 1:2:2 ratio of water, chloroform, and methanol. The layers were allowed to equilibrate, and the aqueous layer was washed 1-3 times with the lower layer of a pre-equilibrated two-phase Bligh-Dyer solution. The aqueous fraction was frozen and lyophilized for analysis.

2.1.1: Solid Phase Extraction (SPE) of BPP-linked Oligosaccharides from Cell Lysates

The lyophilized Bligh-Dyer aqueous phase was resuspended in 10 mM ammonium acetate pH 5.1 with 30% n-propanol (v/v). A C18 solid phase extraction (SPE) column was

equilibrated with the same resuspension solution before the sample was loaded. The wash volume was 80 mL of the equilibration solution, and the elution buffer was increased to 60% n-propanol (Table 1).

Fraction	Vol. (mL)	n-propanol (%)
W	80	30
E1	20	60
E2	20	60
E3	20	60

Table 1: C18-SPE n-propanol gradient method.

Either positive pressure with air or a SPE vacuum manifold were used to speed up the extraction process. The positive pressure method did not work for the larger scale (10 g) bed weight SPE column, and the vacuum manifold was required. The flow rate did not exceed 1 mL/minute to improve the separation efficiency, and the column was not allowed to run dry until the end of the extraction.

2.1.2: LC-MS

All LC-MS analysis was performed on an Agilent 1260 LC with a single quadrupole ESI-MS instrument on a Waters Xbridge Peptide BEH C18 column (4.6 x 50 mm, 3.5 µm). A gradient method of 15-85% n-propanol, with 0.1% ammonium hydroxide as the other solvent,

over 7 minutes was followed by a washing step of 85-95% n-propanol for 3 minutes with a hold at 95% n-propanol for 5 minutes at a flow rate of 1 mL/min.

Species	$[M-H]^{-1}$	$[M-2H]^{-2}$
BPP-tetrasaccharide	1708.9	854.0
c8-BP	845.7	-

Table 2: Selected Ion Monitoring (SIM) m/z ratios for BPP-tetrasaccharide analysis.

2.2: Chemoenzymatic Assembly of Fluorescent CPSA Repeat Unit

The CPSA tetrasaccharide repeat unit was prepared as previously on isolated 2CN-c4-BP.[67, 68] A concentrated stock (10 mM UDP-GlcNAc starting material) of a PglF and WcfR reaction was used to produce the WcfS nucleotide-linked sugar substrate, UDP-AATGal, and was diluted directly in the subsequent enzymatic reactions (500 μM). Similarly, the WcfM reaction was used to produce the WcfN substrate, UDP-Gal*f*, and was coupled directly to the other reactions. Aliquots (8-10 μL) were removed from the 200 μL reactions to monitor each sequential enzymatic step.

2.2.1: Expression of Wcf Enzymes

Chemically competent *E. coli* BL-21(DE3) RIL cells with protein encoding plasmids were cultured from single colonies on LB-agar plates with kanamycin.[7, 68] Cells in 5 mL of LB and kanamycin (50 μg/mL) were grown overnight at 37 °C with shaking and were used to inoculate 0.5 L batches of autoclaved LB. Cultures were grown to an OD_{600} of 0.6-0.8 and protein expression was induced with 1 mM IPTG at 16 °C overnight with shaking.

2.2.2: Isolation of Soluble Proteins

WcfR and WcfM were isolated as previously described with some modifications.[7, 68] Bacterial cells were harvested by centrifugation at 5000 relative centrifugal force (RCF) and 4 °C for 30 min. Cell pellets were resuspended in 0.9% NaCl, transferred to 50 mL Falcon tubes, and pelleted again by centrifugation. Pellets were stored at -70 °C or immediately resuspended in 20 mL of 50 mM Tris-HCl pH 8 (WcfR buffer) or 50 mM potassium phosphate buffer pH 7.4 (WcfM buffer) each with 200 mM NaCl, and cells were lysed by sonication for 3 min (25% amplitude, 1 s on and 1 s off) at 4 °C. Lysed cells were spun at 150000 RCF at 4 °C for 75 min to pellet membrane components and unbroken cells. The supernatant was passed through 3 mL of equilibrated Ni-NTA agarose, and 4 x 3 column volumes of wash buffer (50 mM Tris-HCl pH 8, 200 mM NaCl, and 50 mM imidazole) were passed through the column. The protein was eluted from the column with 6 x 0.5 column volumes of elution buffer (50 mM Tris-HCl pH 8.0, 200 mM NaCl, and 500 mM imidazole). Elutions containing the target protein were dialyzed 1 x 1 L of WcfM or WcfR buffer each with 200 mM NaCl. Proteins were stored at -70 °C.

2.2.3: Isolation of Membrane Fraction (MF) Proteins

Bacterial cells with protein encoding plasmids were harvested and lysed as described above. Unbroken cells were removed by centrifugation at 5000 RCF and 4 °C for 15 min. The supernatant was spun at 150000 RCF for 1 hr at 4 °C, and the pelleted membrane components were resuspended in 1 mL of 50 mM Tris-HCl pH 8 with 200 mM NaCl.

2.2.4: SDS-PAGE and Western Blot of Enzymes

The Ni-NTA column fractions or membrane fractions were analyzed by sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels stained with Coomassie. Proteins were transferred to a nitrocellulose membrane and visualized with Ponceau stain before Western Blot analysis of the vector encoded 6x-His tags. The membrane was washed with deionized water and blocked for 1 hour in a 5% milk solution in phosphate buffered saline pH 7.4 with 0.3% Tween-20 (PBS-T). The nitrocellulose was washed 3 x 15 min in PBS-T with gentle agitation and then incubated with a 1:5,000 dilution of anti-His antibody for 1 hr. The membrane was washed with PBS-T for 30 min before the nitrocellulose was incubated with a (1:20,000) dilution of anti-rabbit antibodies conjugated to alkaline phosphatase (AP). Proteins were detected with NBT/BCIP substrate.

2.2.5: HPLC

Reverse phase HPLC of enzymatic reactions with fluorescent BPs was performed on an Agilent 1100 series HPLC with an Eclipse XDB-C18 column (4.6 x 150 mm, 5 μm) and an isocratic mobile phase of 32% n-propanol and 68% 100 mM ammonium bicarbonate (pH 8.0) at 1 mL/min. A fluorescence detector with an excitation at 340 nm and emission at 390 nm was used to detect fluorescent isoprenoids.

Analysis of nucleotide-linked sugars in the PglF/WcfR reaction and anthranilamide (2AB) labeling reactions with the tetrasaccharide cell lysates were done on a Zorbax Carbohydrate Analysis column (4.6 x 150 mm, 5 μm) with an isocratic mobile phase of 150 mM ammonium acetate pH 4.5 at 1 mL/min. Absorbance at 260 nm was used for nucleotide sugar

detection and a fluorescence detector with an excitation at 330 nm and emission at 420 nm was used for 2AB-labeled sugars.

2.3: C18-MB Immobilization

MagSi-proteomics C18-MBs from Magtivio (20 µL) were washed three times with water in an Eppendorf tube, and aliquots of BPP-tetrasaccharide or 2CN-c4-BPP-tetrasaccharide (20 µL) were added to the washed MBs. The supernatant was magnetically decanted and monitored by LC-MS or HPLC with FLD.

The MBs with immobilized 2CN-c4-BPP-tetrasaccharide were added to a 3% BSA solution (20 µL) for 30 min and decanted. An equal volume of diluted anti-CPSA antibodies in 0.3% PBS-T were added to the MBs for 1 hour and decanted. A stock of FITC-conjugated anti-rabbit secondary antibodies was diluted in 0.3% PBS-T (1:1,000), and 20 µL were incubated with the MBs overnight with gentle agitation in the dark. MBs were aliquoted (4 µL) on nitrocellulose and imaged on a gel imager with a fluorescein filter.

2.4: Fluorescent Labeling of Glycans

Lyophilized glycans from the aqueous Bligh-Dyer extraction (from 5-100 mL of culture) were directly resuspended in hydrolysis solution (50% n-propanol in 1M trifluoroacetic acid (TFA) and hydrolyzed at 50 °C for 15 min in glass tubes and were dried back down. When there was enough sample, the dry weight of the lysates was recorded, and were normalized by dry weight for analysis. Samples were resuspended in labeling solution (acetic acid:DMSO (1:2.3))

and heated at 60 °C for 2-4 hours. For CPSA experiments, the hydrolysis step was skipped, and samples were labeled as previously described for 4 hours.

2.5: Isolation of CPSA from *E. coli*

2.5.1: Extraction and Enzymatic Digestion

The lyophilized Bligh-Dyer aqueous fraction was resuspended in DNase buffer (10 mM Tris-HCl pH 7.5, 1 mM $CaCl_2$, 0.1 mM $CaCl_2$) and placed in a sonication bath for 5 minutes. This fraction was digested with DNase (75 U/L of culture) and RNase (0.625 mg/L of culture) and added directly to the resuspended aqueous fraction overnight at 37 °C; proteinase K (1 mg/L of culture) was added the next day. Polysaccharides in the digested fraction were precipitated with ethanol (80% v/v) overnight at 4 °C, and the precipitate was collected by centrifugation the next day.

2.5.2: Weak Anion Exchange Chromatography (WAX)

CPSA in 5 mL of starting buffer was manually injected onto a GE Healthcare ÄKTAprime™ plus fast protein liquid chromatography system (FPLC) and separated on a weak anion exchange HiTrap DEAE FF (1 mL) column. The starting buffer was 20 mM Tris-HCl pH 8.6 (buffer A) and the elution buffer (buffer B) was 20 mM Tris-HCl pH 8.6 with 0.5 M NaCl. The sample was injected onto a pre-equilibrated column with a 10-minute wash at 100% buffer A. CPSA was eluted with a gradient of 0-100% buffer B for 20 minutes, and B was held at 100% for 17 minutes. Fractions were collected in endotoxin-free Falcon tubes and tested directly by dot blot or lyophilized.

2.5.3: Size Exclusion Chromatography (SEC)

Initial CPSA isolation experiments and 2AB polymer labeling analysis was performed on an Agilent 1100 HPLC system with a PL Aquagel-OH column (7.5 x 300 mm, 8 µm) and an isocratic mobile phase of ultrapure water with 0.02% sodium azide. For CPSA isolation, an absorbance detector at 210 nm was used, but collections were done at pre-determined times and analyzed later by ProQ staining on SDS-PAGE gels or by dot blot analysis. For 2AB labeling analysis, an in-line FLD detector was set at 330 nm and emission at 420 nm to detect 2AB-sugars.

A GE Healthcare ÄKTAprime™ plus FPLC was used for CPSA preparatory chromatography. CPSA was purified by size exclusion chromatography (SEC) with a HiLoadTM 16/60 SuperdexTM 200 column. CPSA was eluted in an isocratic mobile phase of ultrapure water at 1 mL/min for 240 mL total. Fractions were lyophilized and resuspended in 10 mM Tris-HCl pH 7.4 with 15 mM NaCl for analysis. Nucleic acid and protein contaminants were monitored on a NanoDrop spectrophotometer at 260 and 280 nm, respectively. Fractions were analyzed by ProQ Emerald staining and Western blotting.

2.5.4: Dot Blots and ProQ Emerald Staining

Aliquots (2 µL) were dried on a nitrocellulose membrane and blocked with a 5% milk solution in 0.3% PBS-T with rocking at room temperature. The membrane was rinsed three times with deionized water and incubated with 1 mL of anti-CPSA antibodies in 0.3% PBS-T (1:25 dilution) with rocking for 1 hour. The membrane was rinsed twice with 1 mL of 0.3% PBST for 5 min each and was incubated with 1 mL of AP-conjugated anti-rabbit antibodies in 0.3% PBS-T

(1:1,000) for 1 hour. The membrane was rinsed with 0.3% PBST and developed with NBT/BCIP substrate.

Samples were separated on 14% SDS-PAGE gels and stained according to the ProQ Emerald LPS stain kit manufacturer instructions. A CandyCane Glycoprotein ladder was used, and an *E. coli* O55:B5 LPS standard was diluted (1:10) for analysis alongside fractions. The staining protocol was performed continuously but can be stopped after the initial fixing step and stored in the fixing solution before the oxidation and staining process. Gels were stained in the dark.

CHAPTER 3: RESULTS AND DISCUSSION

3.1: Isolation and Hydrophobic Immobilization of the CPSA Repeat Unit from *E. coli*

The first goal of the work in thesis is to hydrophobically immobilize isoprenoid-linked glycans for investigating glycan-interacting partners. One of the first steps in this process is the preparation and isolation of the target glycan. This section will describe the preparation of the recombinant *B. fragilis* CPSA repeat unit expressed in *E. coli*. The Troutman lab has previously demonstrated the function of the Wcf enzymes in the CPSA repeat unit pathways and constructed the tetrasaccharide repeat unit onto a fluorescent BP analog. Once these enzymatic functions were demonstrated *in vitro*, B. Scarbrough constructed a recombinant CPSA production system in *E. coli* for each sequential step in the CPSA repeat unit pathway. Each of the BPP-linked intermediates in cell lysates were identified by a previously established LC-MS method.[37] After the presence of the BPP-tetrasaccharide in this DH5α *E. coli* cell line was confirmed, the next step was to isolate the isoprenoid-linked repeat unit from these cell lysates for immobilization onto C18-functionalized magnetic beads (C18-MBs).

An *E. coli* DH5α cell line with protein-expressing plasmids were used for the LC-MS analysis of BPP-linked intermediates in cell lysates, but we switched to a MG1655 strain with a deletion to improve BPP-tetrasaccharide production and isolation efficiency. This deletion knocked out the *E. coli* polymer: lipid A core ligase WaaL, which commonly appends O-antigen to lipid A. It has also been shown that WaaL can append other O- and K- antigens to lipid A, including non-native glycans.[22-24] Indeed B. Scarbrough has also demonstrated the incorporation of the CPSA repeat unit into this pathway through SDS-PAGE gel analysis of cell lysates with proposed CPSA oligomers appended to lipid A, which were eliminated in ΔwaaL mutants

(unpublished data). While the two *E. coli* cell lines are not isogenic comparisons for comparing production efficiency based solely on the *waaL* deletion, the deletion eliminated alternate lipid-linked oligomeric CPSA glycoforms, and the chosen strain robustly produced BPP-tetrasaccharide (Figure 3). Note that cells were also not normalized by cell numbers and these results did not account for differences in growth between strains.

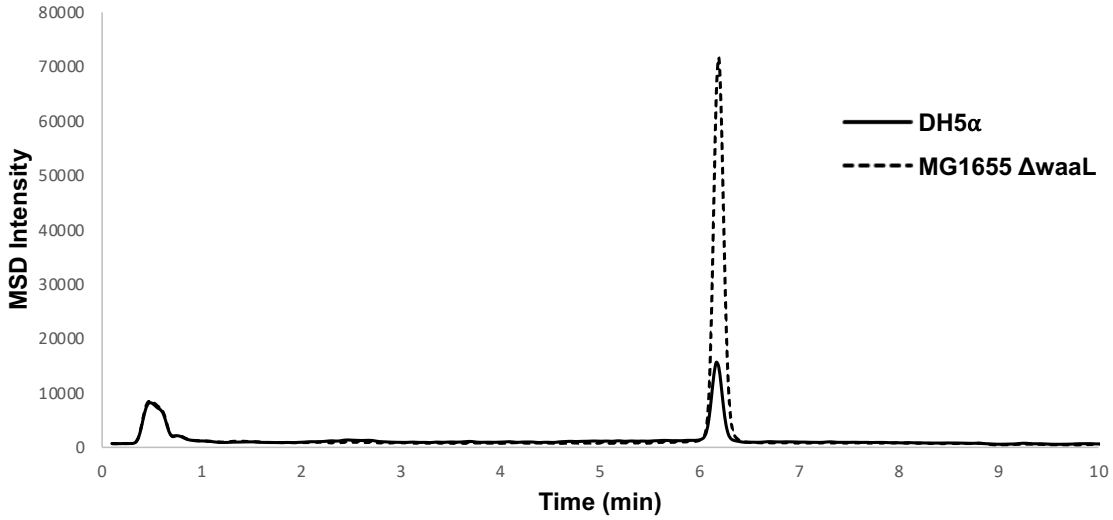

Figure 3: LC-MS reverse phase analysis of the BPP-tetrasaccharide. Selected ion monitoring (SIM) of the $[M-1H]^{-1}$ and $[M-2H]^{-2}$ ion species of the BPP-tetrasaccharide (1708.9 and 854.0 m/z, respectively) from different *E. coli* lysates.

After we chose the *E. coli* MG1655 ΔwaaL cell line for BPP-linked CPSA repeat unit production, we needed to establish an isolation method before immobilization. In particular, the removal of hydrophobic contaminants is important before immobilization onto the C18-MBs. While the proposed C18-MB strategy benefits from site-specific immobilization of the BP lipid anchor, which mimics native glycan presentation, the isolation step is important for the limiting nonspecific interactions of the C18-MB with other hydrophobic components in the sample.

We have previously demonstrated that the BPP-tetrasaccharide partitions to the aqueous phase of a two-phase Bligh-Dyer solvent system, which separates the repeat unit from many hydrophobic contaminants. Another common step in sample cleanup includes solid phase extraction (SPE), so I implemented a C18-SPE step after the Bligh-Dyer liquid-liquid extraction. This strategy was based on previously established methods for the separation of BPP-linked glycans on reverse phase HPLC columns and enabled the separation of cell lysate components by relative hydrophobicity. These conditions were optimized for a shallower gradient of n-propanol (30-60%), and the mobile phase pH was modified to prevent degradation of the silica-based column support. The final elution conditions were determined by LC-MS analysis and are shown below (Figure 4).

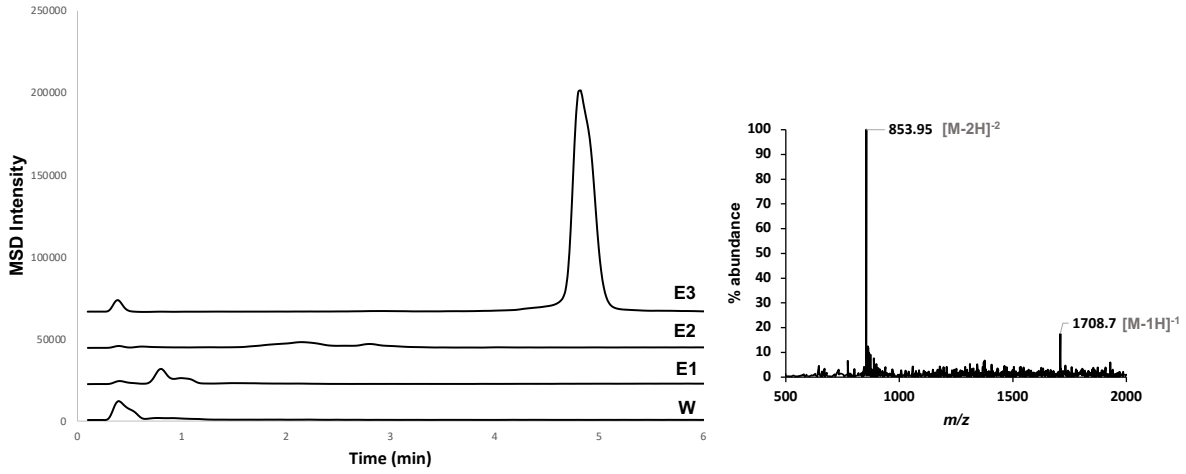

Figure 4: LC-MS reverse phase analysis of C18-SPE fractions. Selected ion mode (SIM) chromatogram for BPP-tetrasaccharide (left) and total ion plot for the peak in E3 (right). Expected m/z ratios for the $[M-1H]^{-1}$ and $[M-2H]^{-2}$ were 1708.9 and 854.0 m/z for the BPP-tetrasaccharide, respectively.

The SPE fraction containing the BPP-linked CPSA repeat unit was incubated with C18-MBs, and the decanted supernatant was analyzed by LC-MS to monitor BPP-tetrasaccharide

immobilization on the C18-MBs (Figure 5). By SIM peak integration, there was an 18% decrease in signal of the BPP-tetrasaccharide after the addition of the MBs, which suggested some of the BPP-linked tetrasaccharide in the sample was immobilized. Notably, there was also a significant decrease in signal from the total ion scan channel (50-2000 m/z) in the 6-11 minute range (Figure 5). Based on the longer retention time on the reverse phase column, these components are relatively more hydrophobic than the BPP-tetrasaccharide, and this data suggested that the C18-MBs removed a significant portion of hydrophobic contaminants from the sample.

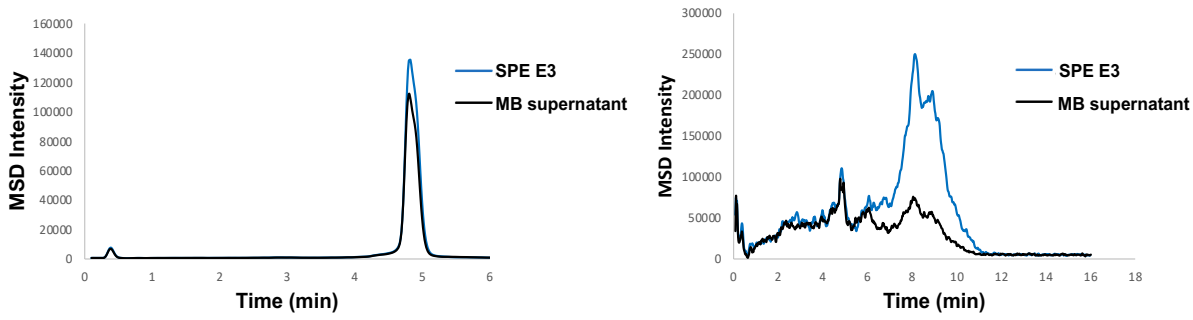

Figure 5: LC-MS analysis of isolated BPP-tetrasaccharide and the supernatant after addition of C18-MBs. SIM channel for the $[M-1H]^{-1}$ and the $[M-2H]^{-2}$ BPP-tetrasaccharide peak (left) and the total ion scan for 50-2000 m/z (right) before (blue) and after (black) the addition of the MBs.

These experiments demonstrated that the BPP-tetrasaccharide can be immobilized onto the C18-MBs, but that the more hydrophobic contaminants saturated a portion of the C18 sites. Notably, more of this peak disappeared after the addition of 50 µL of fresh MBs, and the first MB step helped further purify the BPP-linked intermediate before immobilization. While the SIM peak still did not completely disappear after the addition of the second round of MBs, it is also not clear how much BPP-tetrasaccharide was immobilized, since there are no available comparable internal standards for LC-MS based quantification of the BPP-tetrasaccharide. To

investigate another route for BPP-tetrasaccharide immobilization, I built the CPSA repeat unit *in vitro* on a quantifiable fluorescent BP handle based on previously established methods from the Troutman lab.

3.2: Preparation and Hydrophobic Immobilization of Fluorescent BPP-tetrasaccharide

I first expressed and isolated each of the Wcf enzymes in the CPSA repeat unit pathway and used SDS-PAGE with Western blot analysis to confirm the presence of each His-tagged recombinant protein (Figure 6). To construct the rare sugar UDP-AATGal, I also used a GST-tagged dehydratase PglF in the place of the native UndD2 as previously described.[67]

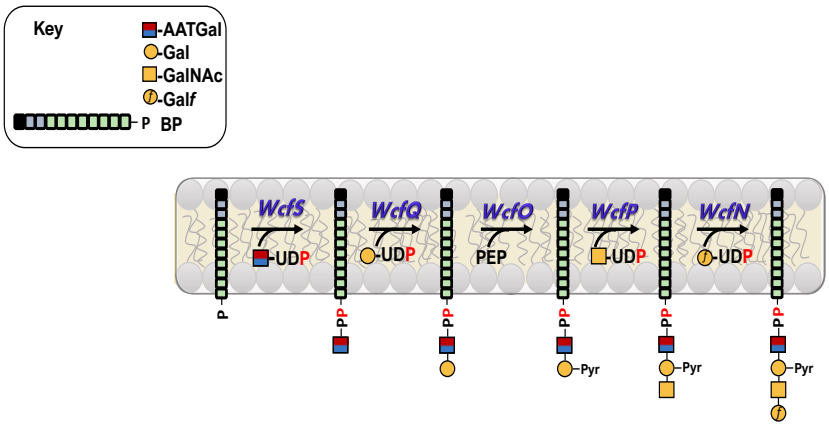

Figure 6: CPSA repeat unit pathway with WcfS, Q, O, P, and N. The nucleotide-linked sugar precursor pathways for UDP-AATGal and UDP-Gal*f* are not shown but were also necessary for BPP-tetrasaccharide assembly.

WcfR was isolated as soluble protein with Ni-NTA purification, and WcfR, S, Q, O, P, and N were isolated as membrane fractions (MF). WcfM was isolated as a MF and as soluble protein, and the initial WcfM soluble sample contained trace amounts of protein in the SDS-PAGE gel (Figure 7) and was not detected by Western analysis (Figure 8). Western blot analysis was particularly important for the MFs because many proteins are present in the cell membrane, and specific detection was required to confirm the target proteins. I repeated this SDS-PAGE gel

for all proteins, and a prominent signal was observed around the expected molecular weight for WcfM (~43 kDa), but I did not repeat the Western for the soluble fraction and continued to functional analysis (Figure 9). The signal observed in the PglF lane by anti-His Western blotting is carryover from the WcfR lane and was not observed in the Western as expected (Figure 8).

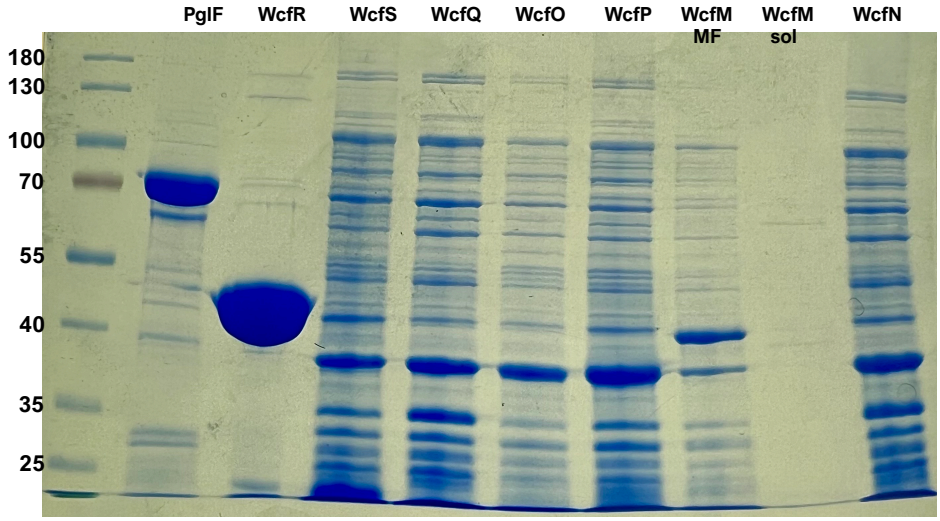

Figure 7: SDS-PAGE gel of PglF and Wcf proteins.

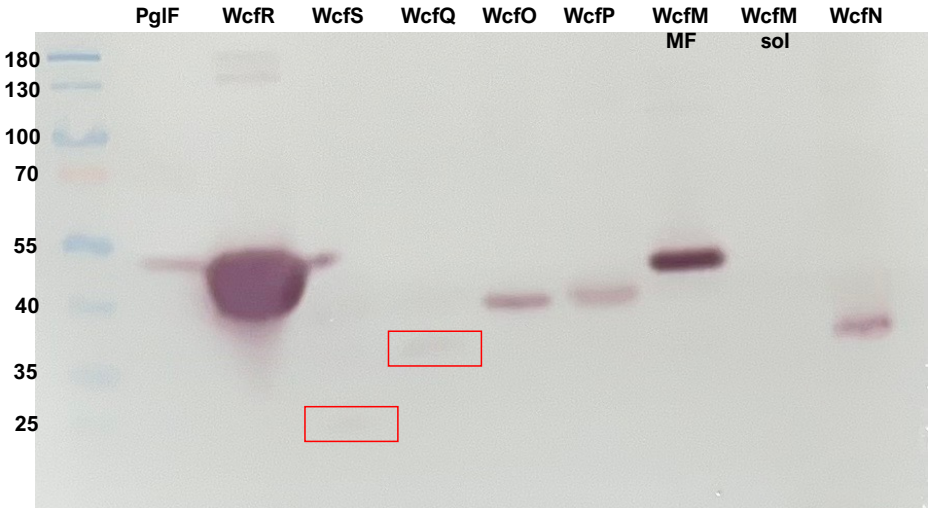

Figure 8: Western blot of PglF and Wcf proteins.

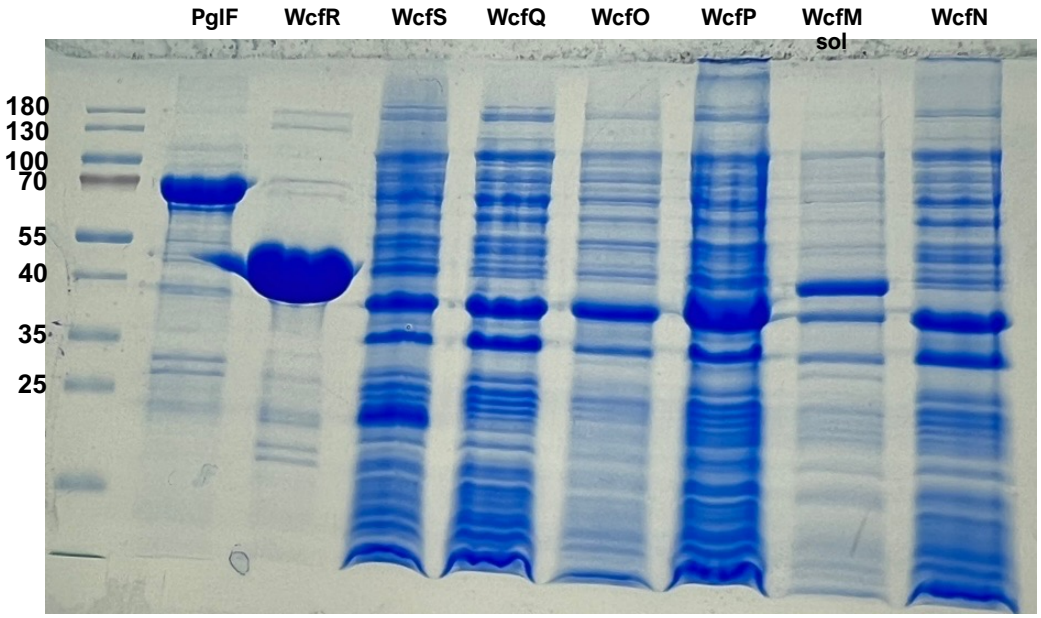

Figure 9: SDS-PAGE analysis of PglF and Wcf enzymes with new soluble WcfM sample.

The proteins on the Western were consistent with the expected sizes (Table 3).

Protein	Size (kDa)	Isolation
WcfR	45	Soluble
WcfS	23	Membrane fraction
WcfQ	32	Membrane fraction
WcfO	40	Membrane fraction
WcfP	44	Membrane fraction
WcfM	43	Soluble
WcfN	34	Membrane fraction

Table 3: Isolated Wcf protein expected sizes. The expected size of proteins was determined from amino acid sequence analysis in Uniprot.

The PglF and Wcf proteins were incubated with respective substrates and built on isolated 2CN-c4-BP (Figure *10*).[67, 68]

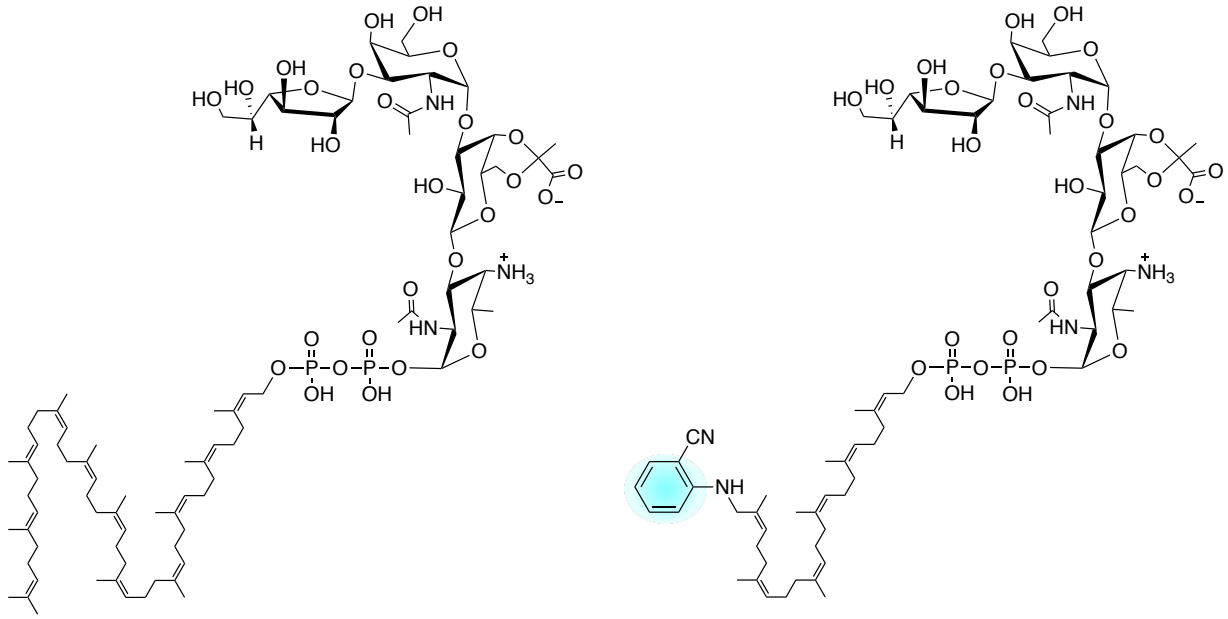

Figure 10: Native BPP-tetrasaccharide and the fluorescent analogue 2CN-BPP-c4-tetrasaccharide structures.

The chemoenzymatic reaction progress was monitored by retention time shifts of the fluorescent BPP-linked intermediates on reverse-phase HPLC. Upon the addition of each enzyme and substrates, there was a respective decrease in the retention time, which was expected as the relative hydrophobicity of the BPP-linked intermediates decreased in each step (Figure 11).

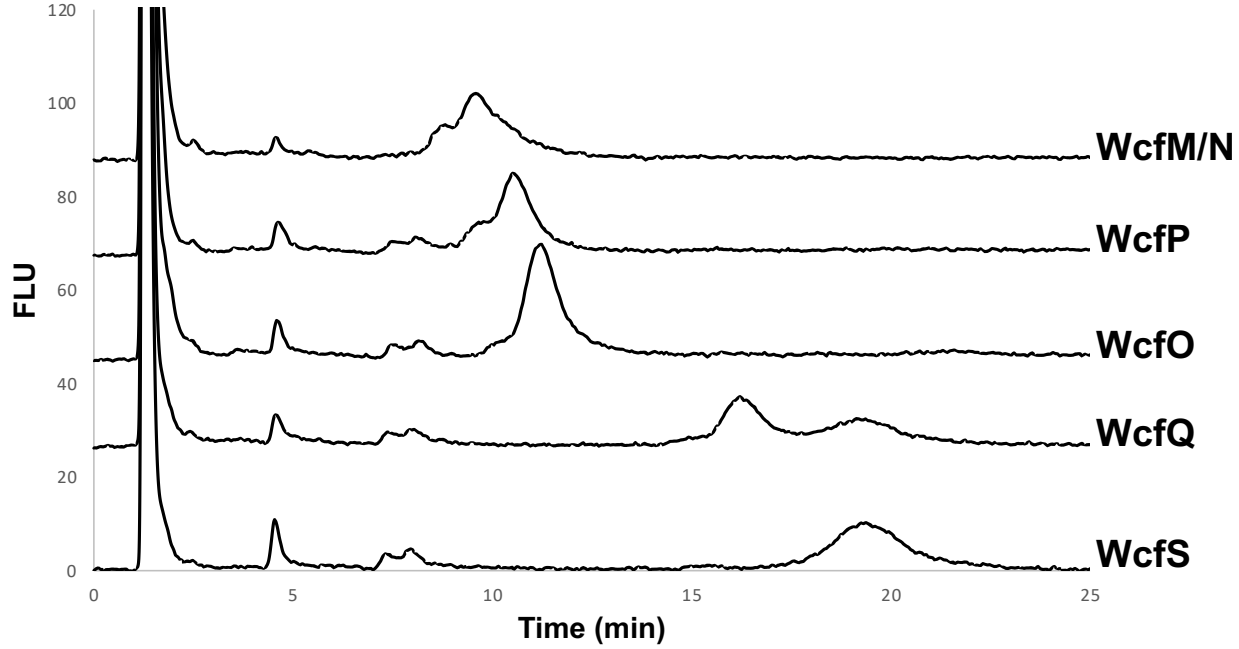

Figure 11: HPLC-FLD reverse phase analysis of the 2CN-c4-BPP-tetrasaccharide. A shift in retention was observed after the addition of each Wcf enzyme in the repeat unit pathway, and the 2CN-c4-BPP-tetrasaccahride product was observed in the WcfM/N reaction.

After the 2CN-c4-BPP-tetrasaccharide product was confirmed, I incubated the reaction directly with C18-MBs and monitored the supernatant by HPLC. The 2CN-c4-BPP-tetrasaccharide fluorescent signal disappeared after the addition of the C18-MBs (Figure 12).

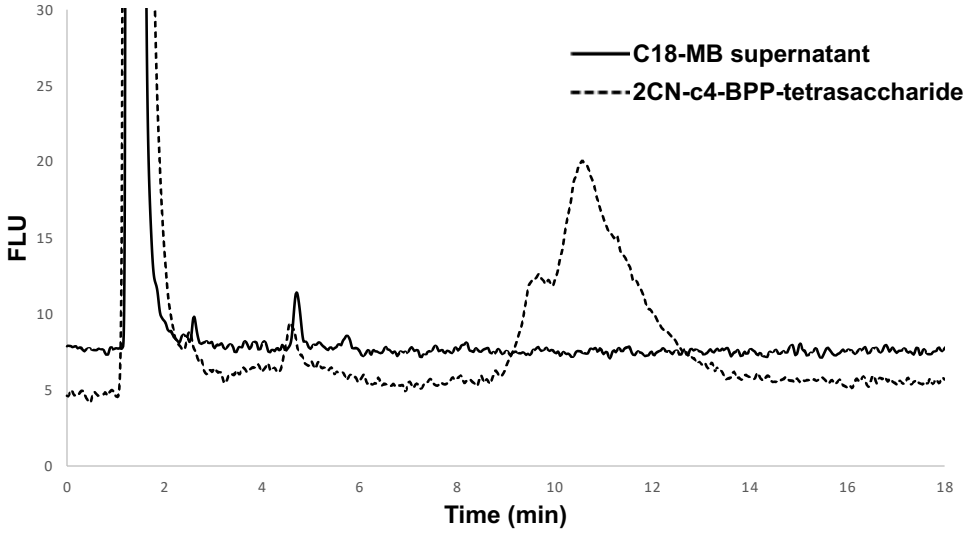

Figure 12: The 2CN-c4-BPP-tetrasaccharide disappeared from the supernatant after the addition of the C18-MBs. This demonstrated that the fluorescent CPSA repeat unit analogue was completely immobilized on the MB.

After the disappearance of the 2CN-c4-BPP-tetrasaccharide signal by HPLC, the MBs were analyzed by a dot blot to detect the CPSA repeat unit of the MB surface. Antibodies were used as model glycan-interacting partners for imaging interactions with the immobilized glycan on the MBs. These polyclonal anti-CPSA antibodies were prepared by Troutman lab member Manoj Dooda from rabbit antiserum adsorbed to CPSA-expressing *B. fragilis* whole cells and selected against ΔCPSA cells provided by Dr. Laurie Comstock. The excess binding sites on the MBs were blocked with a Bovine Serum Albumin (BSA) solution, incubated with anti-CPSA antibodies, and imaged with a fluorescent fluorescein isothiocyanate (FITC)-conjugated secondary antibody. The MBs were imaged on a nitrocellulose membrane and compared to aliquots of 1) only the FITC-conjugated antibodies, 2) unmodified C18-MBs, and 3) blocked C18-MBs incubated with FITC-conjugated antibodies (Figure 13).

As expected, I observed a fluorescent signal (light spot) for FITC-conjugated antibodies, no background noise from the unmodified C18-MBs (black spot), and no signal for the blocked MBs where BSA is meant to prevent FITC-conjugated antibodies from interacting with exposed hydrophobic binding sites on the MBs. Surprisingly, there was no signal for the MBs with the immobilized BPP-tetrasaccharide, which was expected to produce a signal from fluorescent antibody interactions with the CPSA repeat unit on the MB surface.

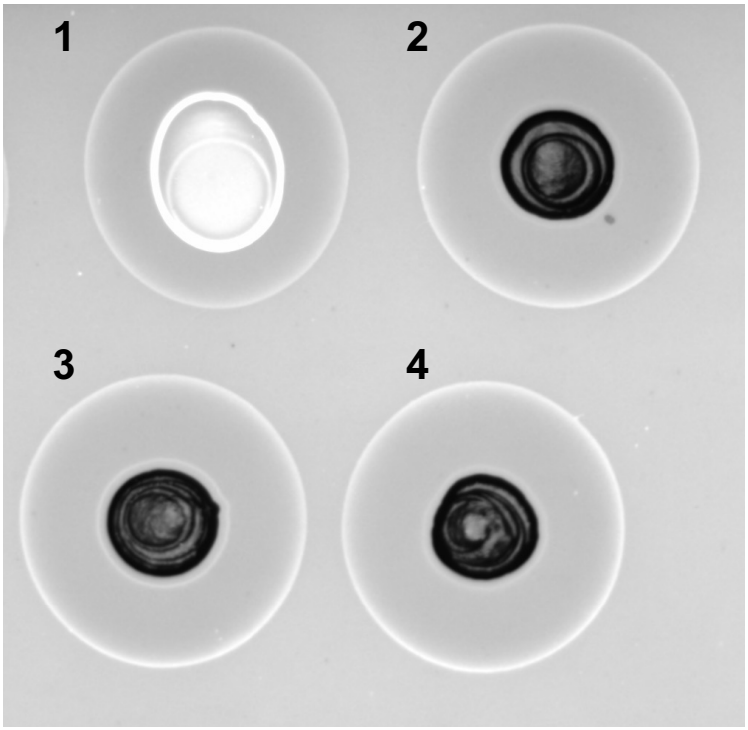

Figure 13: Image of nitrocellulose with 1) FITC-antibodies, 2) C18-MBs 3) Blocked C18-MBs and FITC-antibodies 4) Blocked C18-MBs with 2CN-c4-BPP-tetrasaccharide and antibodies. Light spots indicate a fluorescent signal from the fluorescent secondary antibody, and dark spots are indicative of MBs without detectable association with the fluorescent antibody.

Interestingly, there was also no observable signal for unblocked MBs incubated with FITC-antibodies (not shown). This finding was surprising because these MBs are commonly used to immobilize and capture proteins from solution, so I expected the FITC-conjugated antibodies to interact with the MBs. The antibodies are in a 0.3% Tween in phosphate buffered

saline (PBS-T) solution, while the manufacturer suggested 0.01% Tween detergent. I decreased the amount of Tween-20 in the antibody solution to 0.01% and still did not observe a fluorescent signal from the FITC-conjugated antibodies incubated with unblocked C18-MBs (not shown). However, the 2CN-c4-BPP-tetrasaccharide was immobilized even in approximately 1% Triton-X-100 detergent as indicated by HPLC, so there must be another reason that the secondary FITC-conjugated antibody does not interact directly with the MBs. Nevertheless, the HPLC data suggested that the 2CN-c4-BPP-tetrasaccharide was completely immobilized onto the MB surface.

While it appears that the BPP-tetrasaccharide associated with the C18-MBs by HPLC, it is not clear what the orientation of the immobilized product is on the MB without glycan-binding partner information. An important aspect of the establishment of this C18-MB screening platform is the site-specific immobilization of the lipid anchor because this ensures that the glycan is presented homogenously on the surface for interactions. Additionally, it has not been directly established that the anti-CPSA antibodies interact with a single CPSA repeat unit or how many of the polyclonal antibodies interact with the repeat unit epitope, which may affect the sensitivity of detection.

3.3: A Model Reaction for The Covalent Immobilization of The CPSA Polymer

Our next goal was to establish an immobilization method for the CPSA polymer. The polymer is important because several repeat units are required to elicit certain immune responses, and recognition of these structural aspects may be beneficial in the selection process for the development of glycan-interacting tools.[84] Since immobilization of the free hydrophilic

polysaccharide is not amenable to site-specific noncovalent immobilization on C18-MBs, we aimed to develop an alternative method for site-specific immobilization of CPSA. This method used a common covalent modification method to modify the reducing end of the CPSA polymer via reductive amination, which has been applied in many glycan derivatization methods. During reductive amination, the free aldehyde at the reducing end of the glycan reacts with a primary amine and forms an imine, or Schiff base, intermediate, which is reduced to a secondary amine. While this reductive amination step has been widely utilized to append fluorophores, such as 2-anthranilamide (2AB), to glycans via the reducing end, we proposed the use of a new bifunctional derivative that also has a clickable moiety for immobilization. The strategy of the new proposed immobilization method included a bifunctional labeling reagent (3-benzylazidoaniline), which has a primary amine that reacts with the reducing end of the glycan and leaves a free clickable group that can be used to immobilize the glycan onto DBCO-functionalized MBs (Figure 14).

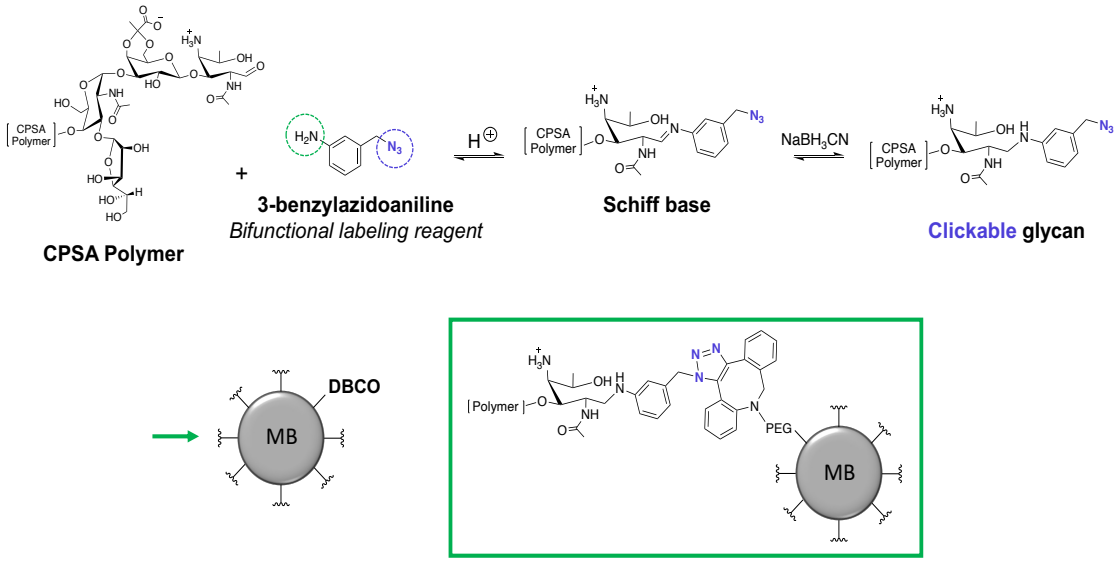

Figure 14. Proposed bifunctional labeling reagent for covalent immobilization of the CPSA polymer. The amine group (circled in green) can react with the free reducing terminus of glycans, while the azide (in purple) can participate in a click chemistry with the magnetic beads.

While the modification to the reducing end of the short CPSA repeat unit results in a loss of structural information that may be important for binding of the tetrasaccharide epitope, we proposed that the relatively small modification at the end of the polymer may be less important for the selection of glycan-binding partners based on total polysaccharide conformation. I first wanted to demonstrate the reaction of the reducing end with a well-known fluorescent labeling reagent, 2AB. I initially used the BPP-tetrasaccharide from cell lysates or isolated from our recombinant CPSA system in *E. coli* to model the reaction with the CPSA polymer since this was compatible with our established isolation methods and analytical techniques. To use the BPP-tetrasaccharide as a model for the reductive amination reaction, I also needed to cleave the BPP lipid anchor and expose the free reducing end of the tetrasaccharide for labeling. To cleave the lipid anchor before reductive amination, I used a previously established method to hydrolyze BPP-linked glycans.[85]

In preliminary fluorophore labeling experiments, there was only one peak associated with the 2AB fluorescent label when the reaction was analyzed on a Zorbax Carbohydrate Analysis column with a 3-aminopropylsilane stationary phase. This data suggested that the concentration of labeled glycans was below the detection limit of the analysis method or that the labeling reaction was not working. Salts and other contaminants can interfere with labeling, so I first tested the labeling reaction in the cell lysate with a glucose standard spiked in. There was complete turnover by the 2AB labeling reagent to 2AB-Glc (Figure 15).

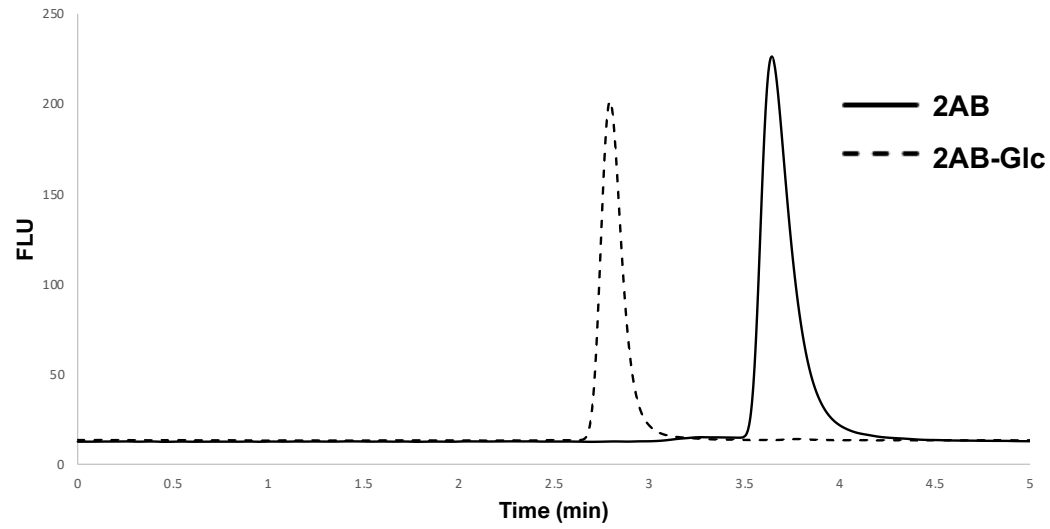

Figure 15: HPLC analysis of 2AB-Glc formation in the cell lysate on a carbohydrate analysis column.

After I increased the amount of starting lysate, there were some smaller peaks but no significant differences in the fluorescent chromatograms between the control lysate, which did not contain the BPP-tetrasaccharide, and the repeat unit-containing lysate (Figure 16).

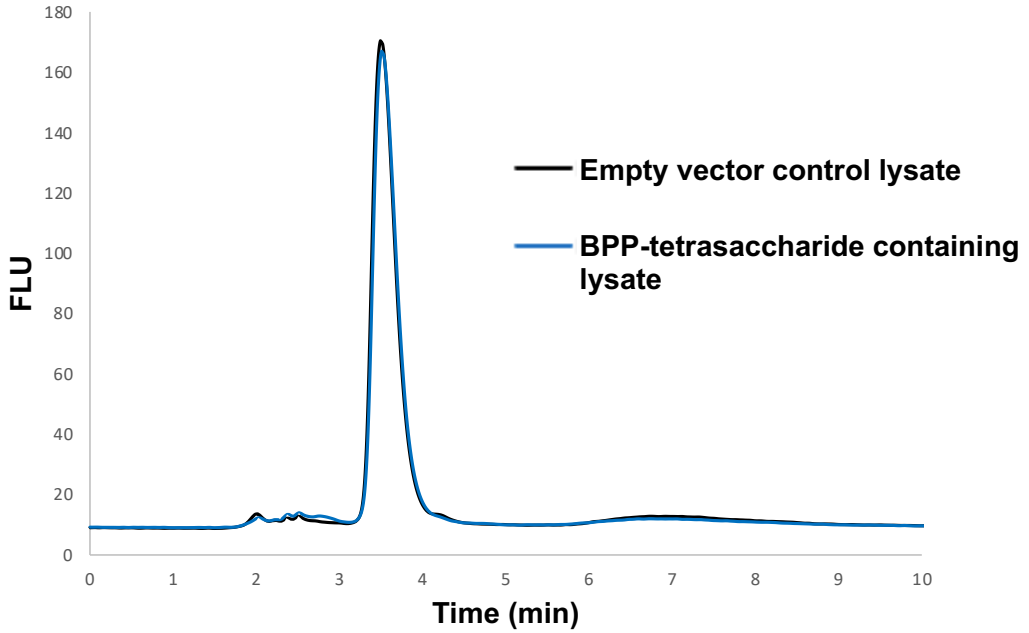

Figure 16: Fluorescent signals in 2AB-labeled BPP-tetrasaccharide expressing cells and empty vector control cell lysates. There is a fluorescent peak at 3.5 min from the excess 2AB labeling reagent, and significant differences between the cell lysates was not observed.

There are some small peaks between 2-3 minutes, which are likely labeled monosaccharides such as 2AB-Glc in the cell lysate. There is also an excess of 2AB labeling reagent at 3.5 minutes, which made this area difficult to interpret. I also monitored the 2AB labeling reaction by LC-MS and observed the disappearance of the BPP-tetrasaccharide after the hydrolysis step, but the expected 2AB-tetrasaccharide product was not readily detected. Interestingly, I also found that the acidic reductive amination labeling conditions can hydrolyze nucleotide-linked sugars to free monosaccharides, which are also present in the cell lysates and can be labeled during the reaction. This was determined by incubating a UDP-Glc standard with 2AB, and a new 2AB-Glc peak was detected by fluorescence (Figure 17).

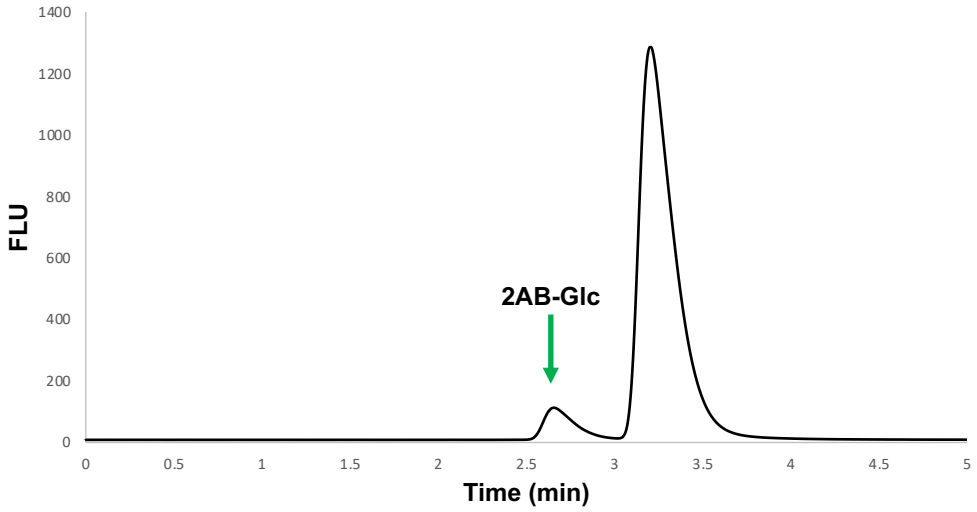

Figure 17: 2AB labeling reaction with UDP-Glc standard. UDP-Glc is hydrolyzed and formed 2AB-Glc under reductive amination labeling conditions.

Since the BPP-tetrasaccharide partitions to the aqueous phase of the Bligh-Dyer extraction, there are interfering components in the sample such as nucleotide-linked sugars and free monosaccharides from the cell lysates. While the 2AB labeling reaction with the Glc

standard worked in the cell lysate, there was no detectable difference between the empty vector control and the BPP-tetrasaccharide containing lysate. This suggested that an additional cleanup step is required before or after labeling to eliminate side products and excess labeling reagent. Labeling the BPP-linked sugar from the organic phase may result in significantly less noise from glycans in the aqueous phase. To accomplish this, I also tried an n-butanol extraction, but the BPP-tetrasaccharide also partitions to the aqueous phase in this liquid-liquid extraction.

With this information, I moved onto the modification of CPSA to see if the labeling results were clearer by analysis of the polymer on a size exclusion chromatography (SEC) column. I skipped the hydrolysis step for cell lysates containing the CPSA polymer and moved straight to the reductive amination step with the 2AB fluorescent labeling reagent. This assumed that the CPSA polymer is in the free polymer form or that it is easily cleaved in the labeling reaction conditions from a phospholipid-linked form. There was a series of unresolved peaks in the 6-10 minute range by SEC analysis on HPLC, and there were no detectable differences between the empty vector control lysate and the CPSA-containing lysate (Figure 18).

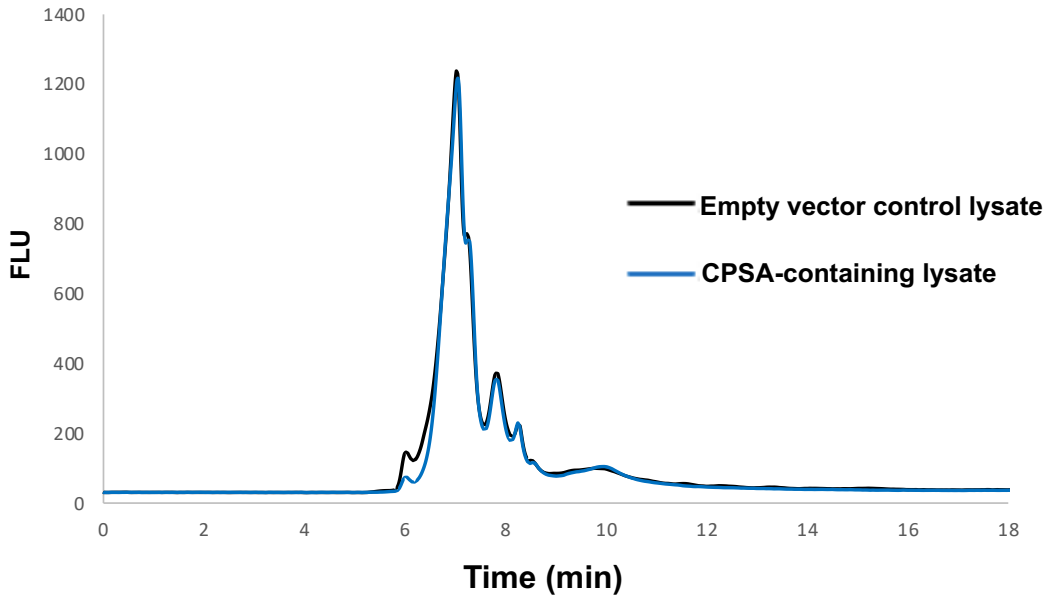

Figure 18: HPLC analysis by SEC of 2AB-labeled cell lysates.

I also tried this labeling reaction in a strain with deletions that knockout the production of other endogenous *E. coli* polysaccharides including the enterobacterial common antigen (ECA) and colanic acid, which may be present in the above chromatogram. Interestingly, there was a new sharp peak in both the control and CPSA-expressing lysate at 10.5-10.6 minutes (Figure 19). Notably, this strain did not contain a waaL deletion, which indicated that CPSA oligomers can also be ligated to lipid A, however if this peak at ~10.5 minutes is indeed LPS, it was significantly smaller in the CPSA-containing strain. While the chromatograms had notable differences compared to the ΔwaaL strains, there were still no clear peaks in the CPSA-expressing strain that were not present in the control.

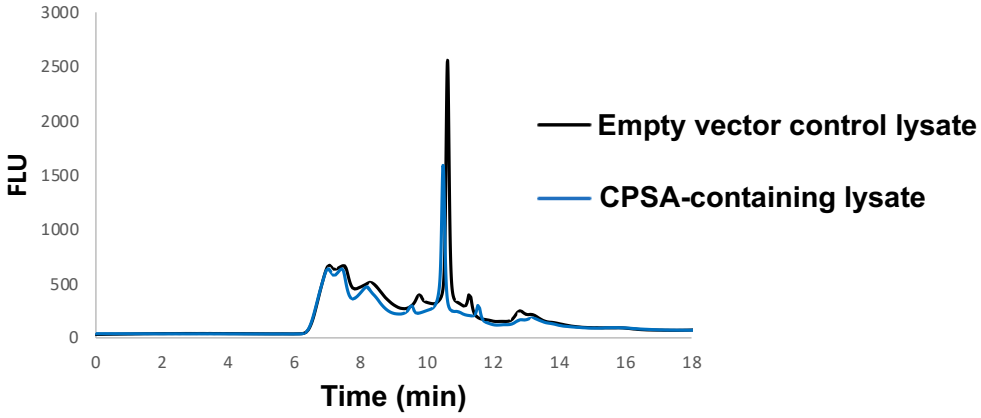

Figure 19: HPLC analysis of 2AB-labeled CPSA-containing strain with deletions of ECA and colanic acid (blue) and empty vector control (black) on a SEC column with FLD. There is a new unidentified peak at 10.5 minutes but minimal differences in the control and CPSA-containing lysate.

3.4: Preliminary Isolation of CPSA Polymer

There are previously established methods for the isolation of the CPSA polymer from the native *B. fragilis* organism. These methods follow a general scheme of chemical bacterial lysis, liquid-liquid extraction followed by dialysis and enzymatic digestion to remove residual proteins and nucleic acids.[20] CPSA in the digested fraction is further purified by chromatography. Size exclusion chromatography (SEC) has been used to separate CPSA from LPS, and ion exchange (IEX) after mild acid hydrolysis separated CPSA from other polysaccharides in the *B. fragilis* capsule. Instead of growing the native organism, which requires anaerobic culturing conditions, I used B. Scarbrough's robust CPSA-expressing *E. coli* strain with a *waaL* deletion to prevent the production of lipid A conjugated to CPSA. This recombinant production system is also convenient because the chosen *E. coli* K-12 laboratory strain does not produce smooth LPS forms due to a mutation that disrupts encoding of *wbbL*, which stops the first committed step of

O-antigen production.[86] While smooth LPS with O-antigen is not produced, the lipid A core is still present in MG1655 *E. coli* and must be removed during purification.

After following the previously established CPSA extraction and enzymatic digestion procedure, I attempted to isolate CPSA by weak anion exchange on a diethylaminoethyl cellulose (DEAE) column with a NaCl gradient. Aliquots from each fraction were analyzed by dot blot analysis to specifically detect CPSA. Each CPSA-containing fraction was analyzed by SDS-PAGE, and purity was assessed by imaging the total polysaccharides in each lane with a ProQ Emerald LPS fluorescent stain kit. Note that CPSA size analysis by SDS-PAGE has been confirmed by Western blot with anti-CPSA antibodies, and the observed bands in the ProQ analysis are consistent with this data. A Coomassie stain was also used to visualize protein contaminants throughout the process (not shown). Lipid A core was effectively removed from the CPSA sample by the detection limits of this analysis, but there were still some lower molecular weight contaminants (Figure 20).

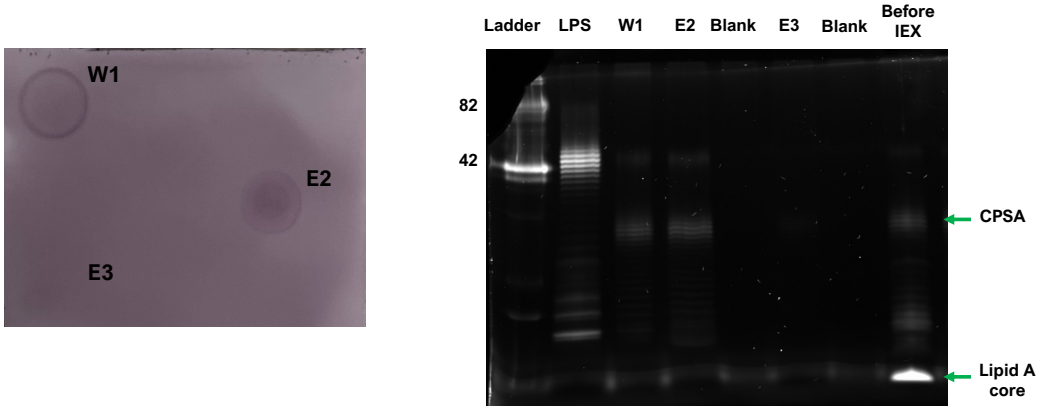

Figure 20: Anti-CPSA dot blot on ion exchange (IEX) fractions (left) and SDS-PAGE of CPSA-containing fractions with ProQ Emerald stain kit (right). Lipid A core is separated from CPSA.

Unlike in the native *B. fragilis* organism, CPSA did not appear to elute as a CPS aggregate, and there are no other similarly sized polysaccharides in the *E. coli* system by SDS-PAGE analysis. In the place of ion exchange chromatography, I next tried a SEC-based CPSA isolation method. I hypothesized that the size difference between the lower molecular weight bands and CPSA may be enough to purify CPSA by SEC without an additional IEX step as done in previous methods.

Each fraction from SEC was lyophilized and analyzed by SDS-PAGE with ProQ staining. I first pooled fractions from multiple injections on a gel permeation chromatography (GPC) analytical HPLC column. While there were CPSA-containing fractions without the previously observed lower molecular contaminants, the amount of isolated CPSA was not enough for NMR analysis of the polymer structure.

CHAPTER 4: CONCLUSIONS AND FUTURE WORK

4.1: Summary – Troutman Lab Work Overview

Bacterial glycans are important virulence and symbiosis factors that mediate a variety of pathogenic and symbiotic interactions with a host. Understanding specific glycan structures is important for developing antibiotics for treating bacterial infections and new therapeutics for diseases. However, there is a lack of highly specific glycan-interacting tools to study, purify, and target bacterial glycans. After isolation of the glycan, the immobilization of this material for screening glycan-interacting partners is often required. The proposed work in this thesis includes two methods for site-specific immobilization that cover different types of bacterial glycans. The first is a noncovalent method, which sought to capture the lipid moiety of BPP-linked glycans onto C18-MBs. The second immobilization method was proposed to covalently modify bacterial polysaccharides via the reducing sugar and link the derivatized polysaccharide to DBCO-MBs via click chemistry. This work stemmed from an extensive combination of culminative work from the Troutman lab and other research groups.

The Troutman lab recently demonstrated that a heptasaccharide virulence factor from pathogenic *C. jejuni* can be chemoenzymatically constructed on an azide-modified analogue of the BP lipid scaffold. This clickable glycan was covalently immobilized via click chemistry on DBCO-MBs, however the chemoenzymatic glycan isolation method requires expensive nucleotide-linked sugars such as UDP-GalNAc. While Troutman and coworkers used a heterologous enzymatic system to convert UDP-GlcNAc to UDP-GalNAc and improved cost efficacy of building the GalNAc-rich heptasaccharide, there is still a need for a generally applicable and cost-effective method for bacterial glycan isolation.

Like the *C. jejuni* heptasaccharide azide-modified BP analogue, the Troutman lab has previously constructed the repeat unit of the *B. fragilis* symbiosis factor, CPSA, on a fluorescent BP analogue. After the functional analysis of CPSA repeat unit enzymes with this analogue, Troutman and coworkers constructed a recombinant glycan production system in *E. coli* for the BPP-linked intermediates and the full CPSA polymer. This glycan production factory is a promising alternative for chemoenzymatic methods because this system can be readily extended to polysaccharides, and *E. coli* cultures are cheap with potential for multigram scale glycan isolation. However, there is a need for generally applicable isolation methods for bacterial glycans from these complex bacterial cell systems.

4.2: Isolation and Noncovalent Immobilization of BPP-linked Oligosaccharides

Since many bacterial surface glycans are constructed on a BP lipid scaffold, the Troutman lab has previously used BP analogues to study and isolate glycans *in vitro*. The work in this thesis extended the use of the ubiquitous lipid scaffold for the development of a generally applicable bacterial glycan isolation method and immobilization strategy for screening new glycan-interacting partners.

Using CPSA as a model system, I established a method for the isolation of the *B. fragilis* BPP-tetrasaccharide in recombinant *E. coli* cell lysates with C18-SPE, which may be applied to other bacterial glycan assembly pathways that utilize the ubiquitous BP lipid anchor. Using the same hydrophobic retention mechanism, the BPP-tetrasaccharide repeat unit was immobilized onto C18-MBs. This system may have distinct advantages over construction of glycans onto

clickable BP analogs because it does not require chemoenzymatic glycan production, which may reduce glycan isolation cost, expedite the development of glycan-interacting tools, and be used for glycan assembly pathways where the enzymes in the pathway cannot be isolated.

While only a relatively small portion of the BPP-tetrasaccharide LC-MS SIM peak disappeared upon incubation with the C18-MBs, it is not clear how much glycan was immobilized due to a lack of comparable standards for quantification by LC-MS. When I extended this to a quantifiable fluorescent 2CN-c4-BPP-tetrasaccharide built chemoenzymatically, I was able to completely immobilize μmol of the 2CN4BPP-tetrasaccharide onto the C18-MBs. This data was promising; however, I was not able to detect the CPSA repeat unit on the MBs with anti-CPSA polyclonal antibodies and fluorescent FITC-conjugated secondary antibodies. This may be because the glycan density on the MB surface is too low for detection, that many or all the anti-CPSA polyclonal antibodies do not interact with a single CPSA repeat unit, or that the glycan is not presented in the proposed site-specific manner via the lipid anchor on the MB surface. This noncovalent BPP-linked oligosaccharide immobilization system will need to be confirmed with a well-characterized glycan-binding partner before it is used for new glycan-interacting partner screening and selection.

4.3: CPSA Isolation and Covalent Immobilization

The isolation of CPSA and other Gram-negative bacterial polysaccharides typically starts with a chemical lysis and liquid-liquid extraction system of hot phenol-water extraction, ethyl ether extraction, and dialysis. This method does not separate the target polysaccharide from LPS and other contaminants in the aqueous phase. In contrast, the Bligh-Dyer single phase solvent

system precipitates LPS, which can be removed by centrifugation. The supernatant can then be converted to a two-solvent system, which separates polysaccharides in the aqueous phase from most endotoxin forms in the organic phase. However, some LOS forms such as lipid A core can partition to the aqueous phase, which we have observed by SDS-PAGE analysis with a ProQ Emerald LPS stain. I was able to remove lipid A core by weak anion exchange on a DEAE column and by size exclusion chromatography. Overall, size exclusion was the more effective method for isolating CPSA from lower molecular weight contaminants but needs to be scaled up for NMR analysis of the recombinant material.

While chemical bacterial lysis is overall more effective than physical sonication used in protein expression, a method for polysaccharide isolation, which avoids the lysis step would be advantageous for simplifying sample complexity and improving isolation efficiency. For instance, there are many proteins, nucleic acids, and small molecules such as nucleotide-linked sugars that are released from the cells during lysis. In contrast, it is relatively more efficient to isolate polysaccharides from the cell growth media. Recently, colanic acid from *E. coli* was readily purified from a modified M9 minimal growth media by precipitation of polysaccharides in the cell media with acetone, centrifugation of precipitate, and dialysis of the resuspension to isolate colanic acid.[49] We have seen that CPSA is excreted into the growth media, and this straightforward purification method can be applied to our system to circumvent the lengthy purification process used in this thesis. Alternatively, the glycan-interacting tools that can be developed from glycan immobilization methods established in this thesis can be used as affinity purification tools for CPSA to avoid lengthy purification.

After CPSA isolation, the next step is to characterize the recombinant material by NMR and to covalently modify the polymer by the reducing end for immobilization onto MBs. The goal of the covalent immobilization system is to modify the reducing end of the CPSA polymer with a bifunctional labeling reagent, which contains an amine and an azide group. The primary amine can undergo reductive amination with the free aldehyde at the glycan reducing end, and the azide specifically interacts with DBCO-MBs to covalently immobilize the glycan.

As a preliminary model for the covalent modification and immobilization of the CPSA polymer, I used the BPP-tetrasaccharide repeat unit in the Bligh-Dyer aqueous phase from cell lysates. In the place of the bifunctional labeling reagent, I used a common fluorescent glycan labeling reagent 2AB, which contains a primary amine and follows the same reductive amination mechanism. To expose the reducing end of the tetrasaccharide, I hydrolyzed the BPP lipid anchor, which has been previously established for other BPP-linked oligosaccharides.[85] The analysis method produced chromatograms with many fluorescent peaks including excess 2AB, and there were not significant differences between controls and the BPP-tetrasaccharide containing samples. I followed the disappearance the BPP-tetrasaccharide SIM signal by LC-MS but did not find convincing evidence for free or labeled tetrasaccharide. This suggested that another cleanup step is required before or after labeling with 2AB. Since there are many glycans in the aqueous BPP-tetrasaccharide-containing Bligh-Dyer fraction, I tried an n-butanol extraction to separate the isoprenoid-linked repeat unit before labeling, but the BPP-tetrasaccharide still partitioned to the aqueous layer. I later found an alternative liquid-liquid extraction, which partitioned the 2CN-c4-BPP-tetrasaccharide to the organic layer, and may be applied to the BPP-tetrasaccharide in cell lysates and deconvolute chromatograms for analyzing

the labeled sugars.[85] There are also commercially available post cleanup kits that can be purchased and used to remove excess labeling reagent.

After cleanup, the reductive amination step may require careful tuning because of the acid-labile pyruvate on the CPSA repeat unit. For instance, the pyruvate can be cleaved in a 5% acetic acid solution at 80 °C for 1 hour.[87] Similarly, I have demonstrated that the acidic reductive amination labeling conditions required for modification of the reducing end can hydrolyze the nucleotide-linked sugar bonds and release free monosaccharides. The potential loss of the pyruvate will need to be monitored during reductive amination to ensure preservation of important structural details because this charged group is crucial to immunological activity and are important for developing effective glycan-interacting tools. Alternatively, mild reductive amination conditions with a methanol-acetate-borate solution have been used to reduce epimerization of hexosamines during labeling and may be a viable alternative to preserve the pyruvate group during modification.[88] Previously, the loss of the pyruvate group was monitored by NMR. While NMR data can give excellent structural information, another method which requires less of the valuable polymer sample for screening labeling reaction conditions is desirable. For instance, Isoelectric Focusing (IEF) may be investigated as viable alternatives to screen for the loss of the pyruvate during labeling.

Alternatively, to the preliminary BPP-tetrasaccharide model, I decided to move straight to CPSA modification with 2AB and analyzed the reaction by SEC analysis on HPLC with FLD detection. I skipped the lipid anchor hydrolysis step for the free polymer and did not see differences between the 2AB-labeled polymer-containing lysate and empty vector control.

Surprisingly, I also did not see a clear 2AB-CPSA peak in a strain with deleted endogenous *E. coli* glycan pathways that eliminate other glycopolymer contaminants (termed the ΔGTase strain). Unlike other isolation and labeling experiments, I did not use a ΔwaaL mutant with the ΔGTase strain, so CPSA oligomers may be sequestered to the lipid A modification pathway and decrease the amount of CPSA polymer available for labeling.

Another route to demonstrate the modification of CPSA via the reducing end by reductive amination for preliminary covalent immobilization experiments includes fluorescence imaging by SDS-PAGE. While I have shown that fluorescently labeled material in an Eppendorf tube is visible using a light at 365 nm after the use of a 10 kDa molecular weight cutoff (MWCO) filter, the 2AB-labeled polymer is not visible in an SDS-PAGE gel. This is likely because the emission wavelength of 2AB is not long enough and is therefore not amenable to sensitive detection by common gel imagers. Alternatively, other dyes including 2-aminoacridone (AMAC) are ideal for fluorescence detection by gel imagers due to longer emission wavelength (525 nm).[89] Since these dyes modify glycans by the same reductive amination modification as the model 2AB and the target bifunctional labeling reagent of this thesis, the use of these dyes can be used to monitor glycan purity and demonstrate labeling of the CPSA polymer via reductive amination.

4.4: Glycan-interacting Partner Assay Development and Selection

Past methods for glycan-interacting partner development are mostly centered around mammalian glycan libraries and glycan-interacting proteins such as lectins and antibodies. The Troutman lab has a unique library of aptamers, which are akin to synthetic antibodies, and we need a system for selecting glycan-interacting aptamers. Current research on aptamers has just

begun to scratch the surface in applying these tools to bacterial molecular targets such as LPS and peptidoglycan. After glycan isolation and selection of the immobilization platform, the next steps include the development of glycan-interacting partner assays with model partners and aptamer selection to expand the library of glycan-binding tools. These glycan-binding partners can be used to isolate, image, and detect bacterial glycans, which are valuable tools for many clinical and research applications in the fields of medicine, microbiology, glycobiology, and immunology.

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