

ABSTRACT

Title of Thesis: CHARACTERIZATION OF A NOVEL
Escherichia coli EXOPOLYSACCHARIDE
AND ITS BIOSYNTHESIS BY NFRB

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Biofilms are made from an association of bacterial cells and extracellular products dominated by a plethora of exopolysaccharides. Accumulating evidence have demonstrated that the bacterial second messenger cyclic-di-guanosine monophosphate (c-di-GMP) promotes the synthesis of these exopolysaccharides through direct allosteric activation of glycosyltransferase enzymes. The *Escherichia coli* inner membrane protein NfrB, which together with the outer membrane protein NfrA acts as a receptor system for phage N4, contains a *N*-terminal glycosyltransferase domain and *C*-terminal c-di-GMP binding domain. Recent research revealed that NfrB is a novel, c-di-GMP controlled glycosyltransferase that is proposed to synthesize a *N*-acetylmannosamine containing polysaccharide product, though the exact structure and function of this remains unknown. Nfr polysaccharide production impedes bacterial motility, which suggests a possible role of the Nfr proteins in bacterial biofilm formation. Here, we carry out *in-vivo* synthesis of novel

Nfr polysaccharide followed by its structural characterization. Preliminary data from MALDI-TOF mass spectrometry and Solid State ^{13}C NMR spectroscopy indicated that the Nfr polysaccharide is mainly a homo polymer of poly- β -(1 $\rightarrow$ 4)-*N*-acetylmannosamine, bound to an aglycone. In addition, we report efforts to develop of a Nfr polysaccharide binding and detection tool, through the mutation of YbcH, a putative Nfr polysaccharide hydrolase enzyme. These studies advance the understanding of Nfr polysaccharide biosynthesis and could offer potential new targets for the development of antibiofilm and antibacterial therapies.

CHARACTERIZATION OF A NOVEL *Escherichia coli* EXOPOLYSACCHARIDE AND ITS
BIOSYNTHESIS BY NFRB

by

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Table of Contents

Acknowledgements.....	ii
Table of Contents.....	iv
List of Figures.....	viii
List of Abbreviations	xiii
Chapter 1: Introduction to Biofilms, Exopolysaccharides and Nfr polysaccharide synthesis by the Nfr system.	1
1.1 <i>Bacterial Biofilms</i>	1
1.2 Exopolysaccharides within the EPS.....	2
1.2.1 Cellulose Exopolysaccharide	4
1.2.2 dPNAG Exopolysaccharide	6
1.3 A novel polysaccharide synthase, NfrB.....	7
.....	9
1.4.1. Biofilm dispersal by Glycosidase enzymes	10
1.5 Importance of the study	12
Chapter 2: Analysis of the NfrB enzymatic glycosyltransferase activity	13
2.1 <i>Introduction</i>	13
2.1.1 A novel Nfr polysaccharide synthase system	13
2.1.2 NfrB requirements for Nfr polysaccharide synthesis.....	15
2.1.3 Physical properties of the NfrB membrane protein	16
2.2 Results and Discussion	17

2.2.1 Expression and preparation of NfrB containing <i>E. coli</i> membrane fractions	17
2.2.2 Assessment of the NfrB enzymatic activity	18
2.2.3 Assessment of the NfrB enzymatic activity dependence on c-di-GMP	19
2.2.4 Identification of a detergent system suitable for the solubilization of NfrB protein from the bacterial membrane fractions	20
2.2.5 Identification of amphiphilic polymer detergents for solubilization of NfrB protein	24
2.2.5 Investigation of the loss of glycosyltransferase enzyme activity in purified NfrB	26
2.3 Conclusions	27
2.4 Experimental Details	28
2.4.1 Expression and membrane fraction preparation of NfrB	28
2.4.2 HPLC-based assay to assess NfrB glycosyltransferase activity	30
2.4.3 Evaluating the c-di-GMP binding dependence of NfrB enzymatic activity in a time-dependent manner	30
2.4.4 Initial Detergent screen and Resolubilization in n-Dodecyl-phosphocholine	31
2.4.5 Amphiphilic polymer screen and Resolubilization in Cyclooctyl acrylamide (COA) – Acrylic Acid (AA) copolymer	32
Chapter 3: Structural characterization of the <i>in vitro</i> produced Nfr polysaccharide	34
3.1 Introduction	34
3.1.1 Polysaccharide biosynthetic mechanisms	34

3.1.2 Nfr polysaccharide biosynthetic pathway	36
3.1.3 Importance of the Nfr polysaccharide structural characterization	36
3.2 Results and Discussion	37
3.2.1 In vitro synthesis and isolation of the Nfr polysaccharide.....	37
3.2.2 Characterization of the Nfr polysaccharide using MALDI-TOF.....	39
3.2.2 Characterization of the Nfr polysaccharide using solid-state ¹³ C NMR....	40
3.3 Conclusions.....	43
3.4 Experimental Details.....	43
3.4.1 Synthesis and ¹³ C solid state NMR analysis of Nfr polysaccharide	43
3.4.2 MALDI-TOF structural analysis of Nfr polysaccharide.....	44
Chapter 4: Development of biological tools to detect the Nfr polysaccharide in bacterial biofilms.	45
4.1 Introduction.....	45
4.1.1 Importance of biofilm exopolysaccharide detection.....	45
4.1.2 Detection of exopolysaccharides in bacterial biofilms	45
4.1.3 Periplasmic protein YbcH and its utilization in developing an exopolysaccharide tool.....	46
4.2 Results and Discussion	47
4.2.1 Expression and purification of recombinant YbcH in <i>E.coli</i> BL21(DE3) cells	47
4.2.2 Fluorescence-based dot blot assay for Nfr polysaccharide identification .	48
4.3 Conclusions.....	49
4.4 Experimental Details.....	50

4.4.1 Expression and purification of YbcH protein	50
4.4.2 Fluorescence-based dot blot assay to test YbcH hydrolytic activity	51
Chapter 5: Summary and future directions	52
Appendix.....	57
Expression and purification WecB protein	57
Bibliography	60

List of Figures

Figure 1.1. The cycle of biofilm development. 1. A planktonic cell irreversibly attaches to a surface. 2. Bacteria multiplication, cellular aggregation and microcolony formation. 3. EPS production. 4. Dispersion of biofilm and detachment of planktonic bacteria.....	2
Figure 1.2. Structure of the di-nucleotide second messenger c-di-GMP.....	3
Figure 1.3. Representative structures of the biofilm exopolysaccharides cellulose, alginate and dPNAG.....	4
Figure 1.4. The structure of the phosphoethanolamine (pEtN) – cellulose repeating unit ¹	6
Figure 1.5. The structure of partially de-N-acetylated poly- β -1,6-N-acetylglucosamine (dPNAG)....	7
Figure 1.6. Graphical representation of the Nfr system and its function in c-di-GMP activated polysaccharide production. NfrB C-terminal MshEN domain binds to c-di-GMP. This leads to an allosteric activation in the NfrB N-terminal glycosyltransferase domain. WecB converts UDP-GlcNAc to UDP-ManNAc which is then utilized for ECA synthesis and for the synthesis of a putative ManNAc polymer by the NfrB GT domain. This gets secreted out by the outer membrane NfrA protein.	9

Figure 2.1. AlphaFold structure of NfrB protein. Highlighted in green is the transmembrane (TM) region, blue is the glycosyltransferase (GT) domain and purple is the c-di-GMP binding MshEN domain.	14
Figure 2.2. Organization of the nfrBA-ybcH operon in <i>E.coli</i> K-12.....	15
Figure 2.3. SDS-PAGE gel and western blot image of NfrB. The ‘lysate’ column shows the proteins present immediately after lysis of the BL21(DE3), the ‘cytosolic fraction’ column shows the components of the isolated cell cytosolic fraction. The ‘membrane fraction’ column shows isolated and concentrated NfrB, which is approximately 87.1 kDa. Anti-His tag antibody has been used for the western blot.	17
Figure 2.4. The consecutive enzymatic reactions occurring in the assay reaction mixture. WecB carries out epimerization between UDP-GlcNAc and UDP-ManNAc. Subsequently, NfrB polymerizes UDP-ManNAc into a putative ManNAc polymer.....	18
Figure 2.5. (a) HPLC traces for NfrB enzyme activity assay reaction mixtures. No WecB and No NfrB reaction mixtures each contain all the components in the test reaction except for WecB and (NfrB + c-di-GMP) respectively. (b) A bar graph plotted using the HPLC data. ...	19
Figure 2.6. Graphs showing the dependence of NfrB polysaccharide production on c-di-GMP and WecB. The results are consistent with c-di-GMP acting to allosterically	

activate the glycosyltransferase domain of NfrB for polysaccharide production, while WecB participates in the epimerization of UDP-GlcNAc into UDP-ManNAc, which serves as the substrate for NfrB activity.	20
Figure 2.7. The structures of the detergents used in the initial detergent screen.....	21
Figure 2.8. SDS-PAGE gel used for the initial detergent screen. N-Dodecyl-phosphocholine (FC-12) has shown the highest NfrB protein resolubilization efficiency in its supernatant, compared to that of the other detergent solutions.....	22
Figure 2.9. Size exclusion chromatogram of NfrB resolubilized in n-Dodecyl-phosphocholine. Depending on the standards sizes which were previously analyzed, fractions 27, 29, 33 and 35 were collected to check for the presence of NfrB protein.....	23
Figure 2.10. SDS-PAGE gel used to analyze the NfrB size exclusion column fractions after n-Dodecyl-phosphocholine resolubilization. NfrB protein was detected in fractions 27 and 29.....	24
Figure 2.11. SDS-PAGE gel analysis used for the initial amphiphilic polymer screen. COA-AA copolymer (Amphipol-18) nanodiscs have shown the highest NfrB protein resolubilization efficiency compared to that of the other polymers.....	25
Figure 2.12. The structure of Cyclooctyl acrylamide (COA) - Acrylic Acid (AA) copolymer.....	26
Figure 2.13. A bar graph plotted using HPLC spectral data, showing that NfrB	

glycosyltransferase activity is only observed while it is in the membrane fraction and is absent when COA-AA nanodiscs are made.....	27.
Figure 3.1. Biosynthetic mechanisms of polysaccharide synthesis and secretion ² , adapted from reference 47.....	34
Figure 3.2. Starting reaction mixture containing c-di-GMP, WecB, MgCl ₂ and UDP-GlcNAc for the synthesis of Nfr polysaccharide.....	37
Figure 3.3. Reaction mixture post-MeOH quenching, Bligh-dyer extraction and Proteinase K treatment.....	38
Figure 3.4. Resulting ManNAc polysaccharide product.....	38
Figure 3.5. (a) MALDI-TOF mass spectrum (Relative intensity vs. m/z) of Nfr polysaccharide after being processed with RStudio and MALDIquant software (b) MALDI-TOF peak intensity vs. ManNAc residue number in a Gaussian distribution.	40
Figure 3.6. solid-state ¹³ C NMR spectra. Top spectrum is for free ManNAc. Bottom spectrum is for the putative ManNAc polymer.....	41
Figure 3.7. Possible Nfr polysaccharide structures.....	42
Figure 4.1. Predicted AlphaFold structure of YbcH protein.....	46
Figure 4.2. SDS-PAGE gel showing HisTrap column purified YbcH which is approximately 32.8 kDa.....	48
Figure 4.3. The result of the fluorescence-based dot blot assay.....	49

Figure 5.1. A possible Nfr polysaccharide synthesis mechanism involving UndPP-GlcNAc as the possible initiator substrate.....	54
Figure 5.2. The interaction between N4 virion and <i>E.coli</i> could be potentially be, initially between gp65 and the Nfr polysaccharide and subsequently between gp65 and NfrA.....	55
Figure 5.3. Graphical representation of the possible outcomes for the mutant strains Δ NfrA, Δ NfrB, Δ WecA and Δ YbcH. Δ NfrA could possibly cause an accumulation of the Nfr polysaccharide in the periplasm. For both Δ NfrB and Δ WecB, a loss of Nfr polysaccharide production altogether could be expected. In the case of Δ YbcH, since there is evidence that YbcH is not required for the synthesis or secretion of Nfr polysaccharide, it's not possible to predict what the outcome would be.	56
Supplementary figure 1.1. SDS-PAGE gel showing HisTrap column purified WecB which is approximately 44.5 kDa.....	58

List of Abbreviations

Extracellular Polymeric Substance (EPS)

extracellular DNA (eDNA)

National Institutes of Health (NIH)

poly N-acetylglucosamine (PNAG)

cyclic di-guanosine monophosphate (c-di-GMP)

Glycosyl transferase (GT)

Transmembrane (TM)

Tetrcopeptide (TPR)

Phosphoethanolamine (pEtN)

de-*N*-acetylated poly- β -1,6-*N*-acetylglucosamine (dPNAG)

Glycosyl transferase 2 (GT2)

N-acetylglucosamine (GlcNAc)

UDP-*N*-acetylmannosamine (UDP-ManNAc)

UDP-*N*-acetylglucosamine (UDP-GlcNAc)

Enterobacterial Common Antigen (ECA)

Glycosyl hydrolase (GH)

Dispersin B (DspB)

glycoprotein 65 (gp65)

Hexahistidine (His₆)

High-Performance Liquid Chromatography (HPLC)

Fast Protein Liquid Chromatography (FPLC)

n-Dodecyl-phosphocholine (FC-12)

Cyclooctyl Acrylamide (COA)

Acrylic Acid (AA)

Phospho-Glycosyltransferase (PGT)

n-Dodecyl- β -D-maltopyranoside (DDM)

n-octyl- β -D-glucopyranoside (β -OG)

n-Dodecyl-N,N-dimethylammonium-N-oxide (LDAO)

Size Exclusion Chromatography (SEC)

Acrylic acid-co-styrene 6-50 (AASTY 6-50)

Diisobutylene-maleic acid (DIBMA)

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

ATP-binding cassette (ABC)

Undecaprenyl Phosphate (UndP)

Undecaprenyl Pyrophosphate (UndPP)

Inner Membrane (IM)

Lipopolysaccharide (LPS)

Matrix Assisted Laser Desorption – Time of Flight (MALDI-TOF)

Mass Spectrometry (MS)

Dihydroxybenzoic acid (DHB)

Mass to charge ratio (m/z)

Magic Angle Spinning (MAS)

Cross Polarization (CP)

Nuclear Magnetic Resonance (NMR)

Phosphate Buffered Saline (PBST)

Bovine Serum Albumin (BSA)

Phospho-ethanolamine modified cellulose (PEC)

Undecaprenyl pyrophosphate N-acetylglucosamine (Und-PP-GlcNAc)

DUF (Domain of Unknown Function)

Glycosyl Hydrolase (GH)

Capsular Polysaccharide (CP)

Chapter 1: Introduction to Biofilms, Exopolysaccharides and Nfr polysaccharide synthesis by the Nfr system.

1.1 Bacterial Biofilms

Biofilms are communities of surface attached bacteria embedded in a self-produced extracellular polymeric substance (EPS)³. This EPS contains a variety of components including exopolysaccharides, proteins, extracellular DNA (eDNA), and lipids and is capable of growing to a thickness of about 0.2 – 1.0 mm⁴⁻⁶. *Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Vibrio cholerae* and *Bacillus subtilis* are just some of the bacterial species that are known to form biofilm as a virulence factor⁷⁻¹⁰. Biofilms are of medicinal and therapeutic significance as acute bacterial infections often manifest from the formation of biofilms associated with pathogenic bacteria¹¹. For instance, biofilm growth in the lungs of cystic fibrosis patients can cause chronic bronchopneumonia, while biofilm growth on indwelling medical devices such as central venous catheters and heart valves can be fatal^{12,13}. According to the National Institutes of Health (NIH) databases, an estimated 65% of all microbial infections and 80% of chronic infections are associated with the formation of biofilms⁴. The reason for this could be the more persistent nature of the biofilm cells compared to planktonic cells, which can, at least in part, be attributed to the protection provided by the biofilm EPS¹⁴.

Biofilm formation occurs in a stepwise manner. As shown in figure 1.1., firstly, free-floating planktonic bacteria irreversibly attach to a biotic or an abiotic surface. Next, multiplication of bacteria results in cellular aggregation and microcolony formation.

Subsequently, the production of a polymer matrix brings about the formation of a mature biofilm. After the biofilm reaches a critical mass, nutrient deficit, unfavorable environmental conditions, metabolite accumulation and quorum sensing leads to degradation of matrix elements, dispersion of the biofilm and liberation of planktonic bacterial cells that spread to other locations where they form new biofilms^{7,12,15,16}. Hence, this cycle can be repeated.

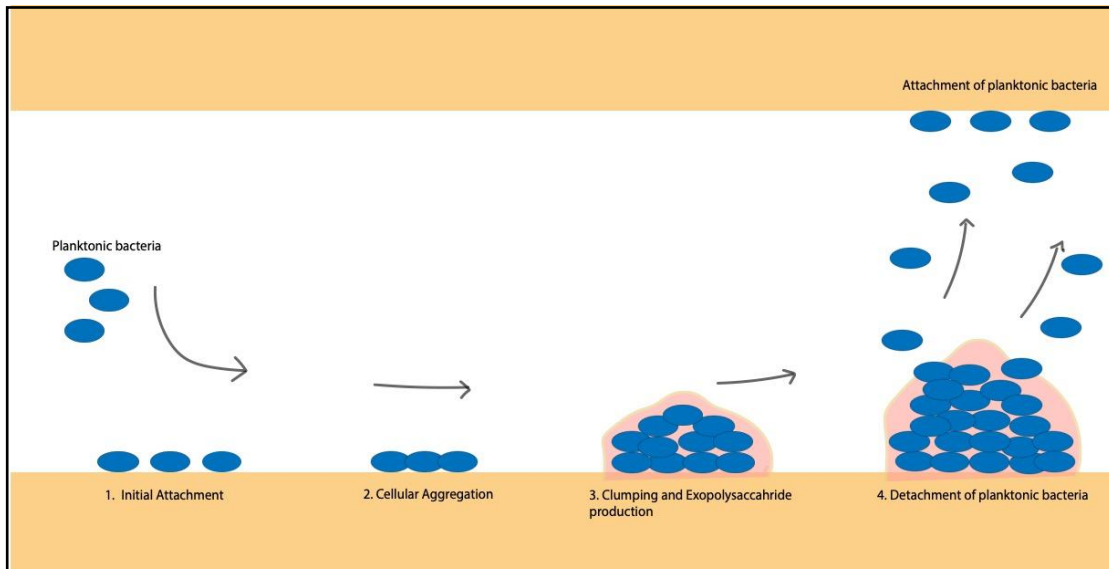


Figure 1.1. The cycle of biofilm development. 1. A planktonic cell irreversibly attaches to a surface. 2. Bacteria multiplication, cellular aggregation and microcolony formation. 3. EPS production. 4. Dispersion of biofilm and detachment of planktonic bacteria.

1.2 Exopolysaccharides within the EPS

The chemical composition of the matrix in each biofilm varies depending on the type of bacteria present as well as the environment in which the biofilm is formed⁵. Of all the different components present within the biofilm EPS, exopolysaccharides

predominate in terms of abundance and play a vital role in the formation as well as the maintenance of the biofilm. Exopolysaccharides provide structural integrity to the EPS allowing formation of the 3-dimensional structure. They assist in cell–cell and cell–surface adherence, restrict the penetration of antimicrobials, and provide collective protection from environmental stresses, such as desiccation, immune effectors or predators^{17,18}. Exopolysaccharides have a global relevance that cannot be overestimated, which is why they are one of the most studied components of the EPS¹⁵. Cellulose, alginate and poly *N*-acetylglucosamine (PNAG) are some commonly observed exopolysaccharides^{11,19}. However, bacteria possess the genetic potential to produce multiple exopolymers at different stages of biofilm development and depending on changing environmental conditions^{5,17}.

The transition from single, planktonic cells to a biofilm-associated community lifestyle is a complex and well-regulated process. In Gram-negative bacteria, the bacterial second messenger cyclic di-guanosine monophosphate (c-di-GMP) (Figure 1.2) plays a key role in combining environmental and cellular signals to regulate this transition by hindering the single cell behavior and favoring biofilm formation¹⁹.

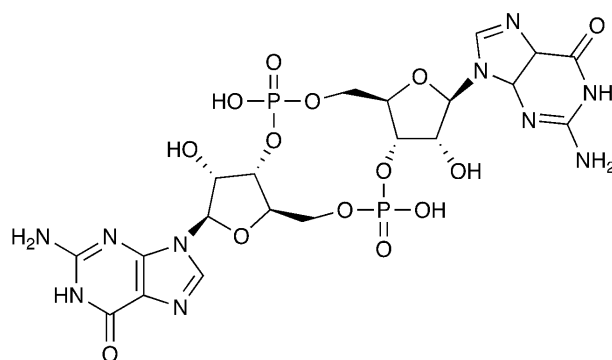


Figure 1.2. Structure of the di-nucleotide second messenger c-di-GMP.

One mechanism by which c-di-GMP promotes biofilm formation is through activation of bacterial exopolysaccharide synthesis¹¹. This is accomplished by direct binding of c-di-GMP to the glycosyltransferase (GT) enzymes responsible for exopolysaccharide biosynthesis, resulting in their allosteric activation¹¹. This is commonly observed in the biosynthesis of several extracellular polysaccharides such as alginate, cellulose and PNAG^{20–22} (Figure 1.3).

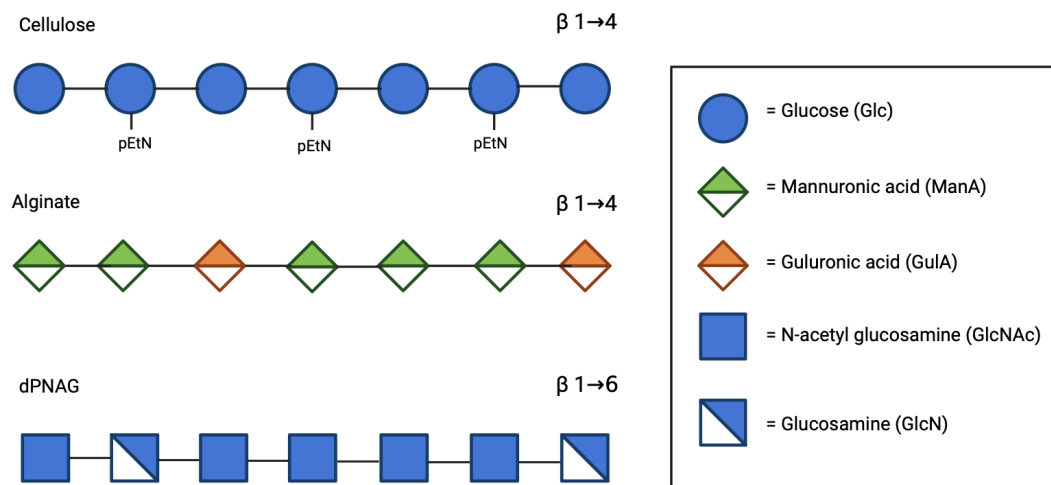


Figure 1.3. Representative structures of the biofilm exopolysaccharides cellulose, alginate and dPNAG.

1.2.1 Cellulose Exopolysaccharide

Cellulose is a glucose homopolymer with β -1,4 glycosidic linkages, that in bacteria is synthesized and secreted by a membrane-inserted cellulose synthase complex²³. This complex typically consists of the proteins encoded by the *bcsABZC* operon²⁴. The function of this protein complex was originally proposed based on its resemblance to other polysaccharide excretion systems²⁴. However, in the course of time, structural

and functional analyses provided verification for the specific protein activities²⁴. BcsA contains eight transmembrane (TM) segments, two cytoplasmic domains, a central glycosyltransferase domain and a C-terminal c-di-GMP binding PilZ region²⁵. During cellulose synthesis, BcsA adds glucose subunits from UDP-glucose onto the growing chain²⁴. This polymerization process is c-di-GMP regulated and has been studied in detail²⁵. BcsB guides the polymer across the periplasmic space towards the outer membrane²⁶. Although BcsA is the catalytically active subunit, the presence of the BcsB subunit is essential both in-vitro and in-vivo catalysis²⁶. In some species, BcsA and BcsB can even be seen fused into a single polypeptide chain²⁶. Usually, the BcsAB enzyme complex exists in an autoinhibitory state with a conserved gating-loop structure interacting with the PilZ region. Then when c-di-GMP binds to the PilZ domain, the active site becomes available for binding the UDP-glucose substrate²⁵.

BcsC contains a C-terminal β -barrel porin domain and N-terminal tetracopeptide repeats (TPR) domains and is believed to assist in the export of the exopolysaccharide across the periplasm and outer membrane²⁴. BcsZ, which is likely a periplasmically localized lipoprotein, has demonstrated cellulose polymer hydrolyzing properties through structural and functional studies²⁴. More specifically, BcsZ belongs to glycoside hydrolase family 8, contains endo-(1 $\rightarrow$ 4)-D-glucanase activity and is believed to contain a signalpeptidase II signal peptide sequence²⁷.

In addition to the *bcsABZC* operon, bacteria often contain additional proteins encoded within the cellulose synthase operon that encode enzymes responsible for modification of the cellulose polysaccharide. For example, the BcsG protein in *E. coli* catalyzes the modification of the cellulose polymer with a phosphoethanolamine (pEtN) group

(Figure 1.4) on alternating glucosyl residues²⁴. This modification allows pEtN-cellulose to assemble into nanocomposites with Curli amyloid fibers in the biofilm extracellular matrix²⁸, which confers cohesion and elasticity to the growing microcolonies and results in biofilms with patterns of wrinkles and high ridges²⁸. Biofilms containing cellulose have been reported in various bacterial species such as *E. coli*, *Agrobacterium tumefaciens*, *Cronobacter sakazakii*, *Acetobacter xylinum* and *Salmonella* spp.¹

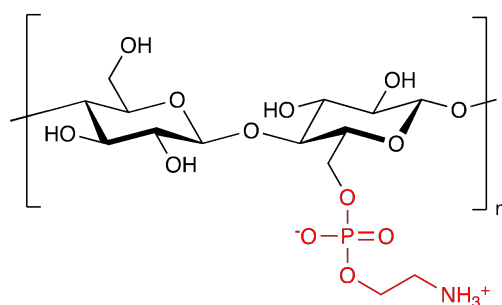


Figure 1.4. The structure of the phosphoethanolamine (pEtN) – cellulose repeating unit¹.

1.2.2 dPNAG Exopolysaccharide

dPNAG is an exopolysaccharide consisting of partially de-*N*-acetylated poly- β -(1 $\rightarrow$ 6)-*N*-acetylglucosamine (Figure 1.5) that is a crucial component of the biofilms of many bacterial species including *S. epidermidis*, *S. aureus*, *E. coli*, *Acinetobacter baumannii*, *Actinobacillus pleuropneumoniae*, *Klebsiella pneumoniae* and *Yersinia pestis*^{29,30}. dPNAG synthesis occurs with the aid of four proteins encoded by the *pgaABCD* or *icaABCD* operons, in Gram-negative and Gram-positive organisms respectively, which

are collectively known as PNAG synthases^{31,32}. The enzyme that is mainly responsible for the biosynthesis of PNAG in Gram-negative bacteria is PgaC, which contains a glycosyl transferase 2 (GT2) domain and carries out the catalysis of the polymerization of *N*-acetylglucosamine (GlcNAc) from the UDP-GlcNAc precursor³¹. PgaC activity is highly dependent on the presence of PgaD. dPNAG biosynthesis is directly regulated by c-di-GMP as follows. C-di-GMP binds to both PgaC and PgaD and forms a stable complex. This binding of c-di-GMP to the PgaCD complex allosterically activates the glycosyltransferase activity of PgaC³¹.

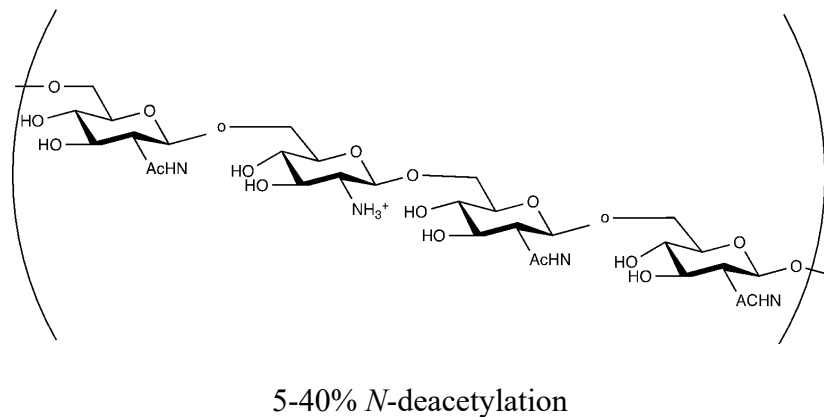


Figure 1.5. The structure of partially de-*N*-acetylated poly- β -1,6-*N*-acetylglucosamine (dPNAG).

1.3 A novel polysaccharide synthase, *NfrB*

In December 2021, a novel, locally c-di-GMP controlled exopolysaccharide synthase known as NfrB was described^{33,34}. This protein was initially identified as a receptor for bacteriophage N4 but was recently revealed to contain a glycosyl transferase family 2 (GT2) domain responsible for production and secretion of a polysaccharide product that impedes bacterial motility. In *E.coli*, NfrB is encoded within the same operon as

NfrA and YbcH, which together form a Nfr polysaccharide secretion system³³ (depicted in Figure 1.6).

This includes the NfrB protein embedded in the inner membrane and NfrA in the outer membrane. NfrA contains a β -barrel porin structure together with a periplasmically localized TPR repeat domain and likely assists Nfr polysaccharide secretion out of the cell. YbcH is a DUF4434 protein that is localized to the periplasm and seems to be playing an auxiliary role in the EPS synthesis and secretion. NfrB contains both a *N*-terminal transmembrane GT2 domain and a *C*-terminal c-di-GMP binding MshEN domain. Furthermore, polysaccharide synthesis by the Nfr system is dependent on c-di-GMP biosynthesis³⁴, suggesting that NfrB activity may be allosterically activated by c-di-GMP in a similar fashion to other biofilm exopolysaccharide biosynthetic enzymes^{20–22}. This c-di-GMP dependence together with the system's ability to hinder bacterial motility led us to hypothesize that the Nfr system functions as a polysaccharide synthesis and secretion system that contributes to *E. coli* biofilm formation.

The only established function of the Nfr polysaccharide so far is serving as a receptor for phage N4³⁴. However, that is not its biological function in *E. coli* and given the prevalence of the *nfr* operon, it is likely to provide some form of growth advantage.

Nfr polysaccharide biosynthesis was shown to be dependent on the cytoplasmic enzyme WecB, which is responsible for the biosynthesis of UDP-N-acetylmannosamine (UDP-ManNAc). WecB, which is also known as NfrC, is a UDP-N-acetylglucosamine 2-epimerase and catalyzes the epimerization between UDP-N-

acetylglucosamine (UDP-GlcNAc) and UDP-ManNAc. This UDP-ManNAc then normally gets converted to UDP-N-acetylmannuronic acid by WecC for the biosynthesis of the Enterobacterial Common Antigen (ECA)³³. WecC is not required for Nfr polysaccharide biosynthesis^{33,34}. Thus, it is likely that the Nfr polysaccharide is composed of ManNAc.

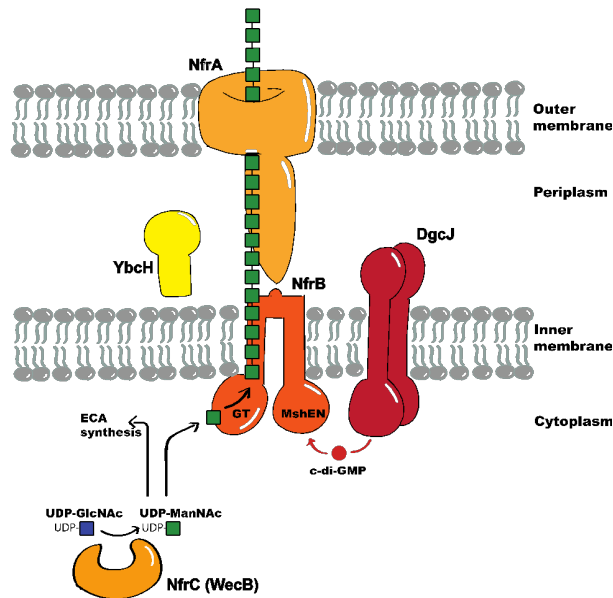


Figure 1.6. Graphical representation of the Nfr system and its function in c-di-GMP activated polysaccharide production. NfrB C-terminal MshEN domain binds to c-di-GMP. This leads to an allosteric activation in the NfrB N-terminal glycosyltransferase domain. WecB converts UDP-GlcNAc to UDP-ManNAc which is then utilized for ECA synthesis and for the synthesis of a putative ManNAc polymer by the NfrB GT domain. This gets secreted out by the outer membrane NfrA protein.

1.4 Treatment of Biofilms

Numerous approaches have been developed to prevent biofilm formation as well as to disperse already formed bacterial biofilms. Utilization of anti-adhesive surfaces, compounds that inhibit the production of functional bacterial adhesins, physical treatments of the biofilm, photodynamic therapy, delivery of signal blockers, interference of biofilm regulation, induction of biofilm detachment and targeting of the biofilm matrix for degradation are some such approaches³⁵. Usage of biofilm dispersal enzymes is one strategy that falls under the method of targeting the biofilm matrix. Extracellular enzymes are capable of dispersing the biofilms by degrading the EPS, more specifically by targeting matrix components such as exopolysaccharides, extracellular DNA and extracellular proteins³⁶. Glycosyl hydrolase enzymes, proteases and deoxyribonucleases are used to target each of these components respectively³⁶.

1.4.1. Biofilm dispersal by Glycosidase enzymes

Glycosyl hydrolases (GHs), enzymes that target and break down the polysaccharide bonds within the biofilm extracellular polymeric substance can be considered as one possible strategy of biofilm dispersal which can be used in therapeutic settings³⁷. The mechanism of action of these enzymes is hydrolyzing the bacterial biofilm by which they initiate the detachment of sessile bacterial cells and transform them into the planktonic state which leads to increased levels of antibiotic susceptibility as well as susceptibility to the host defense systems³⁶. Dispersin B (DspB) hydrolyzes the dPNAG in biofilm EPS using both endo- and exo-glycoside activities³⁶. PgaB is a bifunctional glycoside hydrolase/de-N-acetylase enzyme encoded by the *pgaB* gene in the PNAG biosynthetic operon and contains the ability to degrade dPNAG synthetic analogues³⁶. Apart from these, cellulase that can break down glucose, α amylase that can cleave

amylose, PslG that can cleave the Psl exopolysaccharide and PelA that can degrade the Pel polysaccharide have shown to be useful in biofilm dispersal³⁶.

1.4.2. YbcH as a possible glycoside hydrolase for the Nfr polysaccharide

The periplasmic protein YbcH belongs to the family DUF4434 (Domain of Unknown Function 4434) which is a family of uncharacterized protein from bacteria. Partial structural similarities, when combined with bioinformatic analysis is able to impart indications of function³⁸. This has proven to be true in various cases, particularly with regards to stress- and pathogenesis- related proteins³⁸. On grounds of this, the predicted AlphaFold structure of YbcH was analyzed, and it was revealed that it contains significant structural similarity to the starch hydrolyzing enzyme, beta amylase. Beta amylase belongs to glycosyl hydrolase 14 (GH14) family and exhibits a substrate inverting mechanism in hydrolysis³⁹. Catalytic machinery and molecular mechanism are features that are usually conserved for the vast majority of the glycosidase families. Based on this knowledge, an assumption was made during this study that YbcH could contain potentially similar glycosyl hydrolase activity.

In addition, a feature that can be commonly observed in many of the well-studied biofilm exopolysaccharide synthesis systems including PNAG, cellulose, Psl and Pel systems is that the glycosyl hydrolase enzyme capable of degrading each exopolysaccharide is encoded within the biosynthetic operon. Since *ybcH* is encoded in the same operon as *nfrA* and *nfrB*, this supports its putative function as a Nfr polysaccharide hydrolase enzyme. Based on this assumption, we explored the YbcH protein function. Our preliminary data suggest that YbcH can bind and hydrolyze Nfr polysaccharide. Furthermore, when coupled with a fluorescent protein reporter, it has

the potential to be developed into a Nfr polysaccharide binding and detection tool that can be utilized in biofilm studies.

1.5 Importance of the study

Structure, function, and mechanism of action of the NfrB protein as well as its polysaccharide product remain largely uncharacterized. Given the similarity of the *nfr* operon to other bacterial biofilm polysaccharide biosynthetic pathways, characterization of the enzymes in this pathway would offer potential new targets for the development of anti-biofilm therapies and dispersion agents to be used in the fields of medicine, agriculture, environmental bioremediation as well as in industrial applications. Furthermore, uncovering more information about biofilm EPS could be central to understanding the resilience and the evolutionary success of microbial biofilms in environmental and medical settings.

Chapter 2: Analysis of the NfrB enzymatic glycosyltransferase activity

2.1 Introduction

2.1.1 A novel Nfr polysaccharide synthase system

The bacteriophage N4 is known to infect the gram-negative bacterium *Escherichia coli* K-12⁴⁰. This infection requires specific *E.coli* host cell requirements. During initial investigations of the N4 infection cycle, mapping of the spontaneous *E.coli* K-12 mutants that show resistance to N4 infection was carried out⁴¹. This led to the discovery of the identification of the outer membrane protein NfrA (96 kDa) and the inner membrane protein NfrB (69.5 kDa) as required components for N4 adsorption⁴¹. In addition, NfrC, which is also called WecB, a gene product of an unlinked loci is also required for this adsorption⁴⁰. WecB is a cytoplasmic enzyme and is involved in the Enterobacterial Common Antigen (ECA) exopolysaccharide pathway³⁴.

NfrB is a large protein with a C-terminal c-di-GMP binding MshEN domain and a N-terminal domain that shows similarity to family 2 glycosyltransferase (GT)³³. Incidentally, this GT domain contains conserved DxD, TED and QxxRW active site motifs which are signatures of active GT2 enzymes³³. The C- and N- terminal domains of NfrB are presumed to be connected by two transmembrane helices which anchor NfrB in the inner membrane³³.

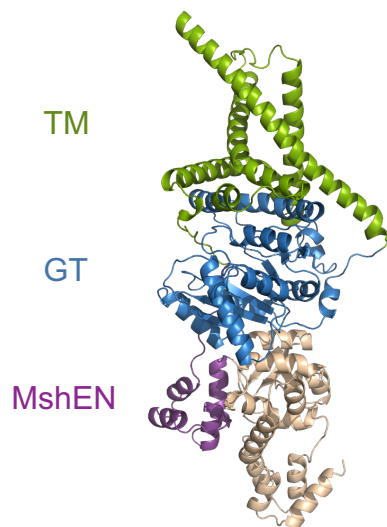


Figure 2.1. AlphaFold structure of NfrB protein. Highlighted in green is the transmembrane (TM) region, blue is the glycosyltransferase (GT) domain and purple is the c-di-GMP binding MshEN domain.

In *E.coli*, the operon that encodes NfrB, (shown in figure 2.2) also encodes two other proteins, namely NfrA and YbcH³⁴. NfrA protein consists a signal sequence, TPR-rich repeats and a sizeable C-terminal region that forms a β -barrels that presumably functions as an outer membrane pore³³. YbcH is a soluble enzyme that is predicted to reside in the periplasmic space³³. It contains a N-terminal signal sequence with a signal peptidase II recognition sequence suggesting that the protein is lipidated and anchored in the inner membrane³³. Collectively, the three proteins NfrA, NfrB and YbcH exhibit the central characteristics of a possible gram-negative polysaccharide synthesis and secretion system³³.

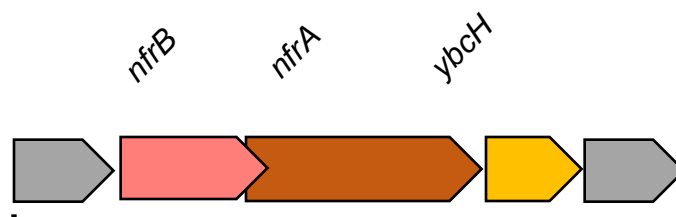


Figure 2.2. Organization of the nfrBA-ybcH operon in *E.coli* K-12.

Previously it was believed that the N4 glycoprotein 65 (gp65) interacts with *E.coli* ECA during adsorption⁴¹. However, more recent studies show that the bacteriophage N4 in fact does not target the ECA as the primary target but instead targets a novel surface polysaccharide³⁴. In December 2021, NfrB, which was previously only identified as a receptor for bacteriophage N4 was reported by two simultaneous studies as the synthase enzyme that gives rise to this novel polysaccharide^{33,34}. This discovery opened doors for researchers for a whole new area of studies.

2.1.2 NfrB requirements for Nfr polysaccharide synthesis

The presence of the MshEN-like domain in NfrB suggests that its activity could be allosterically regulated by c-di-GMP³⁴. It has been shown that NfrB specifically binds c-di-GMP with a K_d of $1 \pm 0.35 \mu\text{M}$ and shows local activation³³. Previous data supports that c-di-GMP binding to NfrB stimulates polysaccharide secretion and this c-di-GMP is supplied predominantly by the diguanylate cyclase enzyme DgcJ³⁴. On the other hand, phosphodiesterase PdeL acts to reduce local c-di-GMP levels and represses the polysaccharide synthesis process³⁴.

Apart from c-di-GMP, the cytoplasmic protein NfrC (also known as WecB) is also required for Nfr polysaccharide biosynthesis. WecB is one of the enzymes of the ECA exopolysaccharide pathway³⁴. Even though it was previously believed that ECA is

required for N4 phage adsorption, it was later revealed that only one of the components from the ECA biosynthetic gene cluster, the *N*-acetylglucosamine 2-epimerase enzyme WecB, is required for N4 phage infection³⁴. WecB is specifically involved in UDP-ManNAc biosynthesis, suggesting that the novel Nfr polysaccharide is a polymer containing ManNAc monomeric units³⁴.

2.1.3 Physical properties of the NfrB membrane protein

NfrB is a membrane protein and is therefore hydrophobic. As is common with most membrane proteins, this hydrophobicity makes any structural and functional characterization challenging as they require detergents or other solubilizing agents to facilitate their extraction from the native lipid membrane⁴². There are various methodologies that have been developed to aid in membrane protein research.

Detergents are an invaluable tool in membrane protein isolation and purification. Detergents are amphipathic molecules with a polar head group and a hydrophobic tail group⁴³. They spontaneously form spherical micellar structures that can solubilize membrane proteins⁴³. Detergents solubilize these membrane proteins by generating micelles that mimic the natural lipid bilayer environment that the protein normally resides in⁴³.

Nanodiscs are another significant tool in protein studies. They provide membrane proteins with a bilayer surface of defined composition and structure that can act as a nanometer scale machinery in active protein extraction processes⁴⁴.

2.2 Results and Discussion

2.2.1 Expression and preparation of NfrB containing *E. coli* membrane fractions

The *E. coli nfrB* gene fused with a C-terminal hexahistidine tag (His₆-NfrB) was cloned into a pET21 vector and expressed in BL21(DE3) cells. These cells were grown and *nfrB* expression was induced with IPTG. This was followed by cell lysis and inverted membrane vesicles (IMVs) containing NfrB were floated on a sucrose cushion and isolated to as NfrB containing membrane fractions. As seen in Figure 2.3, NfrB protein was identified in crude cell lysates and in the isolated membrane fraction, but no significant NfrB protein was present in the soluble cytosolic protein fraction.

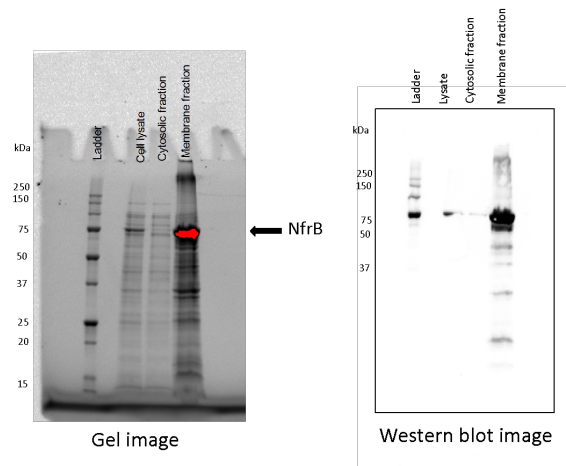


Figure 2.3. SDS-PAGE gel and western blot image of NfrB. The ‘lysate’ column shows the proteins present immediately after lysis of the BL21(DE3), the ‘cytosolic fraction’ column shows the components of the isolated cell cytosolic fraction. The ‘membrane fraction’ column shows isolated and concentrated NfrB, which is approximately 87.1 kDa. Anti-His tag antibody has been used for the western blot.

2.2.2 Assessment of the NfrB enzymatic activity

A High-Performance Liquid Chromatography (HPLC) based assay was developed to test the NfrB enzymatic activity. It is hypothesized that NfrB catalyzes the polymerization of UDP-ManNAc to form a putative poly-ManNAc polymer. In that reaction UDP is released as a byproduct. UDP-ManNAc is not commercially available, so we employed the enzyme WecB to generate UDP-ManNAc in situ from UDP-GlcNAc. An overview of these reactions are shown in Figure 2.4.

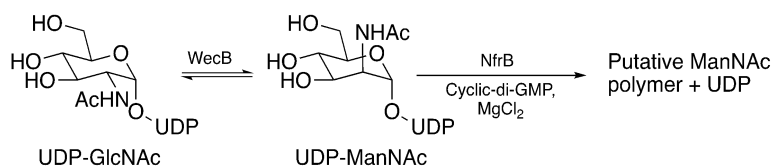


Figure 2.4. The consecutive enzymatic reactions occurring in the assay reaction mixture. WecB carries out epimerization between UDP-GlcNAc and UDP-ManNAc. Subsequently, NfrB polymerizes UDP-ManNAc into a putative ManNAc polymer.

In a typical assay, NfrB membrane fractions were incubated in the presence or absence of c-di-GMP and WecB protein for an appropriate amount of time and then the reactions were quenched by adding an equivalent volume of methanol. The concentration of UDP formed in the assay was assessed by HPLC. Based on the HPLC chromatograms, both c-di-GMP and WecB are required for the optimum activity of NfrB (Figure 2.5).

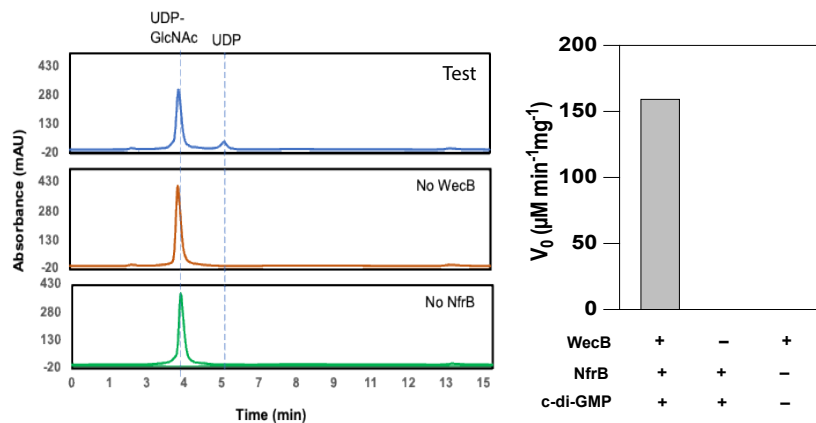


Figure 2.5. (a) HPLC traces for NfrB enzyme activity assay reaction mixtures. No WecB and No NfrB reaction mixtures each contain all the components in the test reaction except for WecB and (NfrB + c-di-GMP) respectively. (b) A bar graph plotted using the HPLC data.

2.2.3 Assessment of the NfrB enzymatic activity dependence on c-di-GMP

We next carried out a series of time-dependent assays to determine NfrB dependence on c-di-GMP and WecB. As seen in Figure 2.6, NfrB activity was only observed when both WecB and c-di-GMP were present in the reaction.

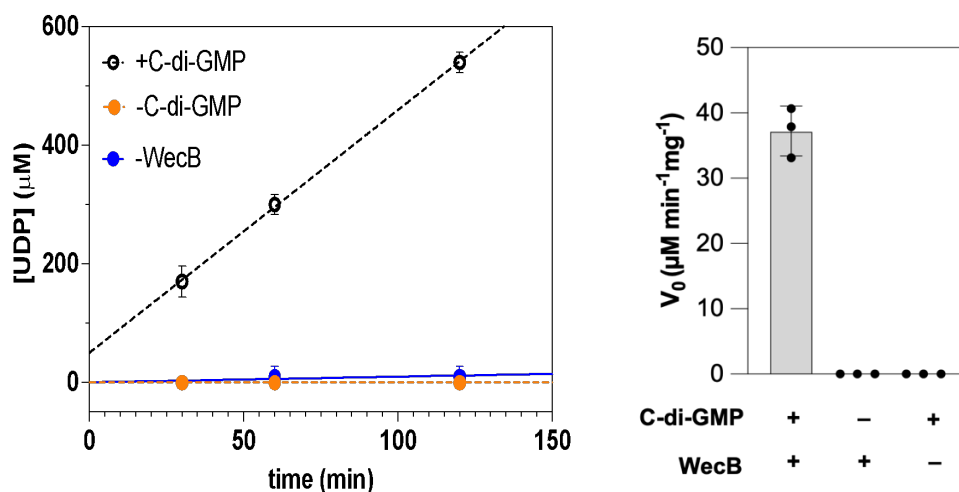


Figure 2.6. Graphs showing the dependence of NfrB polysaccharide production on c-di-GMP and WecB. The results are consistent with c-di-GMP acting to allosterically activate the glycosyltransferase domain of NfrB for polysaccharide production, while WecB participates in the epimerization of UDP-GlcNAc into UDP-ManNAc, which serves as the substrate for NfrB activity.

2.2.4 Identification of a detergent system suitable for the solubilization of NfrB protein from the bacterial membrane fractions.

Isolating membrane proteins from the membrane fraction is complicated by their insolubility in water. Therefore, detergent and amphiphilic polymer screens were carried out to identify suitable candidates for the resolubilization of NfrB from *E. coli* membrane fraction.

An initial detergent screen was carried out using a panel of commonly-used detergents, including n-Dodecyl- β -D-maltopyranoside (DDM), n-Dodecyl-phosphocholine (FC-12), n-octyl- β -D-glucopyranoside (β -OG) and n-Dodecyl-N,N-dimethylamine-N-

oxide (LDAO) (Figure 2.7). These are either zwitterionic or neutral detergents and do not cause protein denaturing.

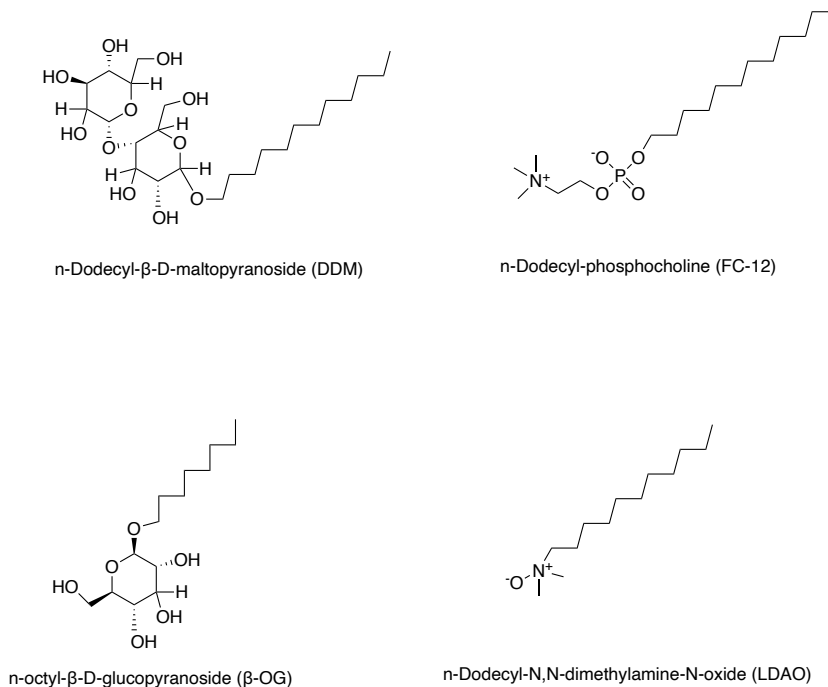


Figure 2.7. The structures of the detergents used in the initial detergent screen.

According to the results shown in Figure 2.8, DDM FC-12, and LDAO were all able to partially resolubilize NfrB from *E. coli* membrane fractions, with FC-12 having the highest solubilization efficiency. Thus, FC-12 was picked as the most suitable candidate for further detergent resolubilization of NfrB from *E.coli* membrane fractions.

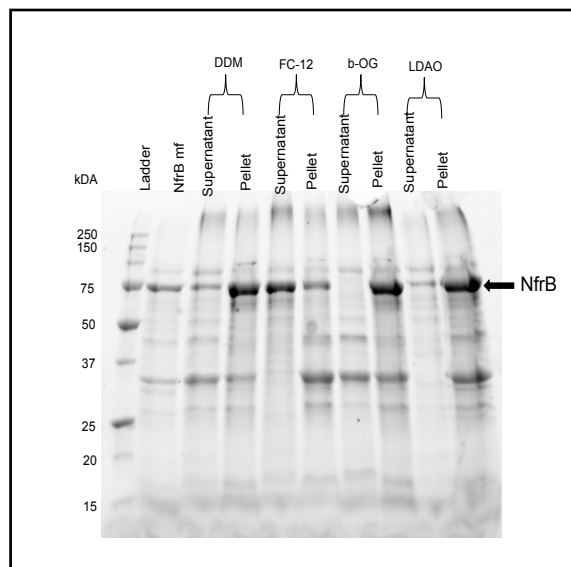


Figure 2.8. SDS-PAGE gel used for the initial detergent screen. N-Dodecylphosphocholine (FC-12) has shown the highest NfrB protein resolubilization efficiency in its supernatant, compared to that of the other detergent solutions.

2.2.5 Detergent resolubilization of NfrB protein using FC-12

For FC-12 detergent resolubilization of NfrB, 1% (w/v) FC-12 was added to *E.coli* membrane fraction containing NfrB at a final protein concentration of 5 mg/mL. After incubating for 1 hour at the samples were centrifuged to remove insoluble membrane components. IMAC chromatography was used to isolate the his-tagged NfrB protein as a

pure 87.1 kDa protein, as observed on SDS-PAGE gel. Size exclusion chromatography measurements, as seen in Figure 2.9 and 2.10, were used to confirm that the FC-12 resolubilized NfrB remains soluble and is not forming larger aggregates. The protein was found to elute as a major peak between 31-33 min (fraction 29 & 30), with smaller peaks at 30 min (fraction 27) , 35 min (fraction 33) and 37 min (fraction 35). Fractions

27, 29, 33 and 35 from the SEC were collected and analyzed using 12% SDS-PAGE to assess the presence and purity of NfrB.

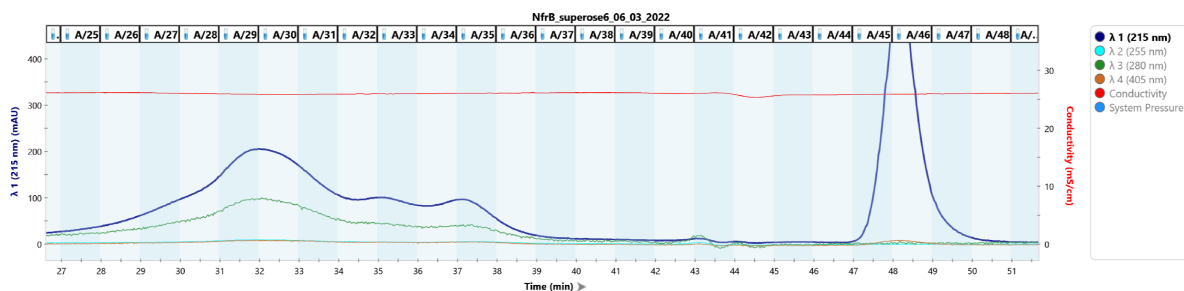


Figure 2.9. Size exclusion chromatogram of NfrB resolubilized in n-Dodecylphosphocholine. Depending on the standards sizes which were previously analyzed, fractions 27, 29, 33 and 35 were collected to check for the presence of NfrB protein.

As seen in figure 2.10, the SEC fractions 27 and 29 were shown to contain NfrB, with the majority of the protein being found in fraction 29. Based on the retention time of molecular weight (MW) standards, the protein eluting in fraction 29 is predicted to have a MW of ~150 kDa. This is close to what we would expect for the molecular weight of a NfrB monomer (87.1 kDa) within a FC-12 micelle (~30-60 kDa)⁴⁵. However, a NfrB activity assay revealed that this purified NfrB has lost its glycosyltransferase enzymatic activity.

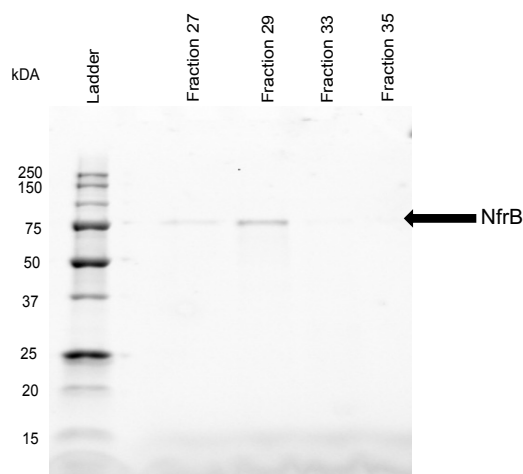


Figure 2.10. SDS-PAGE gel used to analyze the NfrB size exclusion column fractions after n-Dodecyl-phosphocholine resolubilization. NfrB protein was detected in fractions 27 and 29.

2.2.5 Identification of amphiphilic polymer detergents for solubilization of NfrB protein

Amphiphilic polymeric detergents have previously been demonstrated to resolubilize membrane proteins into native lipid nano-discs⁴⁶. An initial screen of commercial detergents including cyclooctyl acrylamide – acrylic acid (COA-AA), acrylic acid-co-styrene 6-50 (AASTY 6-50), styrene maleic acid copolymer 300 (SMALP 300) and diisobutylene-maleic acid modified with glucosamine (DIBMA-GlcN) as the candidates. Each polymer was incubated with NfrB membrane fraction containing 40 mg/mL protein at a final concentration of 1% (w/v), and then the resolubilized proteins samples were separated from the insoluble material through centrifugation.

As can be seen in Figure 2.11, the COA-AA was able to efficiently resolubilize NfrB protein into nanodiscs that were purified by IMAC on Ni-NTA resin. This COA-AA copolymer, shown in Figure 2.12, is amphipathic and has been used to isolate purified

membrane proteins in native lipid nanodiscs^{46,47}. However, a NfrB activity assay revealed that NfrB purified using this method has no enzymatic activity as can be seen in Figure 2.13.

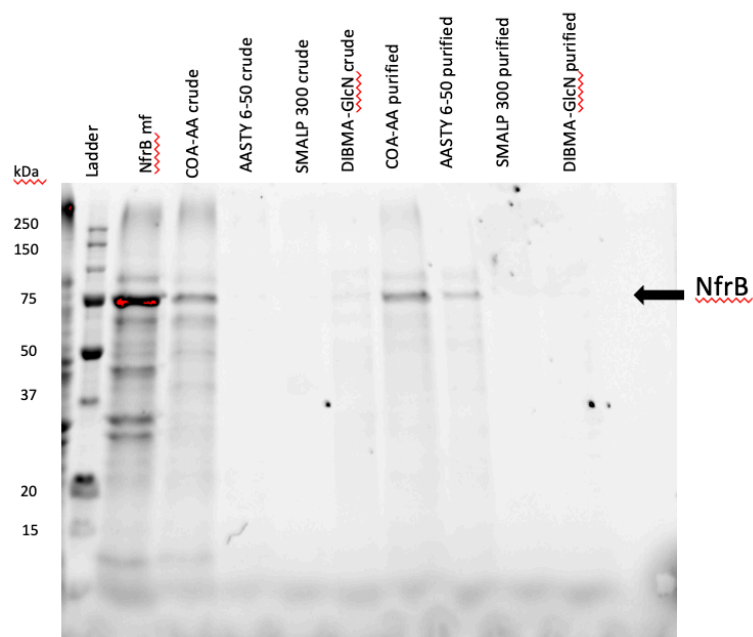


Figure 2.11. SDS-PAGE gel analysis used for the initial amphiphilic polymer screen. The soluble fraction (lanes 3-6) and Ni-NTA purified nanodisc (lanes 7-10) are shown. The COA-AA copolymer nanodiscs show the highest NfrB protein resolubilization efficiency.

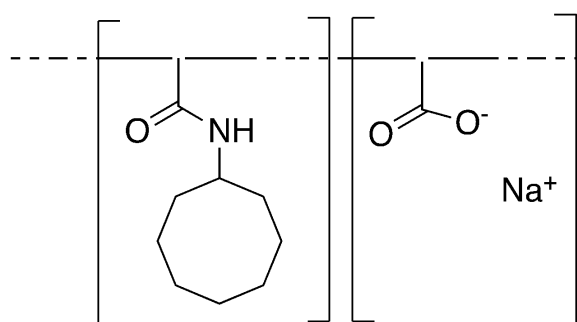


Figure 2.12. The structure of Cyclooctyl acrylamide (COA) - Acrylic Acid (AA) copolymer.

2.2.5 Investigation of the loss of glycosyltransferase enzyme activity in purified NfrB

Based on the western blot and gel-permeation chromatography results, it appears that NfrB polymer nanodiscs contain folded and non-aggregated protein. Thus, it is our hypothesis that the loss of Nfr polysaccharide production by purified NfrB is due to a loss of a membrane associated acceptor substrate required to initiate the polysaccharide biosynthesis. To ascertain the step at which NfrB loses its enzymatic activity during COA-AA nanodisc formation, several assays were carried out using NfrB protein from different stages of the extraction and resolubilizing process (Figure 2.13). NfrB containing membrane fractions, polymeric detergent extracted membrane fractions, and purified NfrB polymer nanodiscs were used as the enzyme source for these assays.

According to the results, UDP formation and therefor NfrB activity was only observed in NfrB containing membrane fraction. Moreover, as seen in Figure 2.13, UMP formation was also observed in addition to UDP formation. This was an interesting observation since, UMP formation in NfrB extracted into the polymeric detergent was significantly greater than that of NfrB in *E. coli* membrane fraction. UMP formation is usually associated with phospho-glycosyltransferase (PGT) enzyme activity⁴⁸. PGTs

are enzymes that transfer a sugar-1-phosphate onto an undecaprenyl-phosphate acceptor and are often required as the initiating step for the biosynthesis of common bacterial cell surface carbohydrates, including lipopolysaccharide *O*-antigens, capsular polysaccharides (CPS), enterobacterial common antigen (ECA), and glycoprotein. These observations suggest that UDP-GlcNAc PGT enzyme activity is present in the membrane fraction, and that this protein activity is resolubilized separately from NfrB protein during COA-AA nanodisc formation. Together, these results suggest that a UDP-GlcNAc dependent PGT enzyme activity may be required to prepare the initiating substrate for NfrB activity.

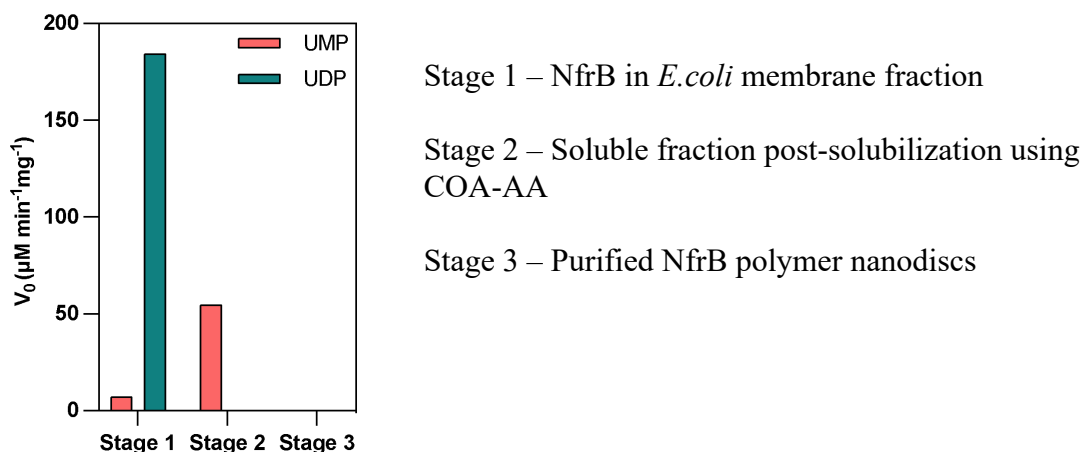


Figure 2.13. A bar graph plotted using HPLC spectral data, showing that NfrB glycosyltransferase activity is only observed while it is in the membrane fraction and is absent when COA-AA nanodiscs are made.

2.3 Conclusions

NfrB is a protein with an approximate size of 87.1 kDa and is mainly present in the membrane fraction of *E. coli* cell lysate that is responsible for the biosynthesis of a

putative ManNAc polysaccharide. The synthesis of the putative ManNAc polymer occurs via a two-step enzymatic process. In the first step, UDP-GlcNAc is isomerized to form UDP-ManNAc by WecB and in the subsequent step, UDP-ManNAc is polymerized into the ManNAc polymer by the NfrB protein activity. In addition to the putative polymer, UDP is generated during this process. NfrB requires c-di-GMP to carry out the polymerization process successfully.

When initiating the reaction from UDP-GlcNAc, NfrB activity is dependent on both WecB and c-di-GMP being present in the reaction. C-di-GMP acts to allosterically activate the glycosyltransferase domain of NfrB for polysaccharide production, while WecB participates in the epimerization of UDP-GlcNAc to form UDP-MANNAc, which serves as the substrate for NfrB activity.

n-Dodecyl-phosphocholine (FC-12) can be used for the detergent solubilization of NfrB protein from the bacterial membrane fractions, and COA-AA copolymer can effectively isolate purified NfrB in native lipid nanodiscs. However, both result in pure yet inactive protein. Moreover, in the bacterial membrane fraction containing NfrB there is also UDP-GlcNAc PGT enzyme activity present.

2.4 Experimental Details

2.4.1 Expression and membrane fraction preparation of NfrB

A DNA sequence for the expression of *Escherichia coli* NfrB containing a C-terminal hexahistidine (His₆-NfrB) was synthesized, codon optimized for expression in *E. coli* and cloned into a pET21 vector using NdeI and XhoI cleavage sites to give a

pET21:NfrB plasmid construct. BL21(DE3) cells transformed with this plasmid were grown overnight at 37 °C with shaking in 50 mL of LB broth containing 100 µg/mL ampicillin. 5 mL of overnight culture was transferred to 500 mL of fresh LB broth containing 100 µg/mL ampicillin and grown to OD₆₀₀ of 0.6-0.8, then induced with IPTG to a final concentration of 1 mM.

The cultures were then grown overnight at 17 °C with shaking. After this, the cell pellet was harvested by centrifugation for 20 minutes at 5500 rcf and 4 °C. Cell pellets could be stored at -20 °C before lysis. The cell pellet was resuspended 10 mL/1 g in PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4) and lysed by sonication (30% amplitude, 10 seconds pulse, 30 seconds pause, for a total sonication time of 2.5 minutes. This was repeated 3 times). The cell debris was pelleted for 20 minutes at 13,000 rcf and 4 °C. The lysate was then floated on a 6 mL 1.8 M sucrose cushion and centrifuged for 2 hours at 60,000 rcf and 4 °C. Under these conditions, the bacterial membrane fraction accumulates on top of the sucrose cushion. Thereby the centrifugation results in a bottom sucrose layer, a middle layer containing the membrane fraction and a cytosolic fraction-containing uppermost layer. The middle layer out of the three layers was then separated. The membrane fraction was diluted to with 5 volumes of PBS buffer and then the NfrB membrane fraction was pelleted by centrifugation for 1 hour at 60,000 rcf and 4 °C. The membrane pellet was suspended in MOPS buffer (25 mM MOPS, 300 mM NaCl, 10 % glycerol v/v, pH 7.5) to a final protein concentration of 40 mg/mL. A 12% agarose gel and a western blot using anti-His tag antibody were carried out to confirm the presence of the NfrB protein. The purified enzyme was stored at -80 °C until use.

2.4.2 HPLC-based assay to assess NfrB glycosyltransferase activity.

Individual 50 μ L reaction mixtures containing MOPS, 5 mM MgCl_2 , 5 mM UDP-GlcNAc, 0.5 μ M WecB, and 2 μ M c-di-GMP were prepared. To these was added 10 μ L of 5.5 mg/mL of NfrB membrane fraction to initiate the reaction. After incubating for an hour at room temperature, the reactions were centrifuged for 2 minutes at 17,000 rcf. 20 μ L of the reaction supernatant was separated and was mixed with an equal volume of methanol. This together with 100 μ L of MiliQ water was added into HPLC glass vials and HPLC was carried out using linear gradients of Buffer A (20 mM KH_2PO_4 , 2 mM tetrabutylammonium bisulfate, pH 6) and Acetonitrile. Buffer A was run at 100% for 1 minute followed by a linear gradient from 100 % to 50 % over 7 minutes, from 50 % to 40 % over 3 minutes and from 40% back to 100 % over 1 minute.

2.4.3 Evaluating the c-di-GMP binding dependence of NfrB enzymatic activity in a time-dependent manner.

A series of HPLC-based activity assays were carried out. To a 100 μ L reaction mixture, MOPS buffer, 5 mM MgCl_2 , 5 mM UDP-GlcNAc, 0.5 μ M WecB, 2 μ M c-di-GMP and MiliQ water were added. This was mixed and spun for 2 minutes at 17,000 rcf. 20 μ L of NfrB enzyme (5.5 mg/mL) containing membrane fraction was then added to this mixture. To test the dependence of activity on c-di-GMP and WecB, control reactions were carried out in the absence of c-di-GMP and WecB respectively. The reactions were incubated at room temperature. 20 μ L of the reaction mixture was separated at 30 minutes, 1 hour and 2 hours' time intervals. HPLC was carried out using each of these separated fractions. The added substrate concentration was calculated using the amount

of UDP-GlcNAc added. After HPLC runs, each HPLC spectrum was individually analyzed. UDP-GlcNAc and UDP get eluted at different time points as separate peaks. The area under each peak is measured and the percentage conversion of UDP-GlcNAc into UDP is calculated. Using this value and the initially added UDP-GlcNAc concentration, the concentration of the generated UDP is determined.

2.4.4 Initial Detergent screen and Resolubilization in n-Dodecyl-phosphocholine

An initial detergent screen was carried out using the detergents n-Dodecyl- β -D-maltopyranoside (DDM), n-Dodecyl-phosphocholine (FC-12), n-octyl- β -D-glucopyranoside (β -OG) and n-Dodecyl-N,N-dimethylamine-N-oxide (LDAO). DDM (1 % w/v), FC-12 (1 % w/v), β -OG (6 %, w/v) and LDAO (0.5 %, w/v) solutions were prepared and in 1 mL of each of these *E. coli* membrane fraction containing NfrB was solubilized to a final protein concentration of 5 mg/mL. The 4 mixtures were incubated for 30 minutes with shaking, and then centrifuged for 1 hour at 60,000 rcf and 4 °C. The pellets were separated from the supernatants and resuspended in 180 μ L of PBS buffer. The protein content in each of the pellets and supernatants were assessed using 12 % SDS-PAGE.

According to the result, n-Dodecyl-phosphocholine (FC-12) was picked as the most suitable candidate for the detergent resolubilization of NfrB from *E. coli* membrane fraction.

After the initial screen, during FC-12 detergent resolubilization of NfrB, 2 mL of (1%, w/v) FC-12 was used and *E.coli* membrane fraction containing NfrB was solubilized in it to a final protein concentration of 5 mg/mL. This was shake incubated for 1 hour

followed by spin centrifugation for 1 hour at 60000 rcf and 4 °C. The supernatant was separated and then purified via FPLC on a HisTrap column via a linear gradient from Buffer A (25 mM Sodium Phosphate, 150 mM NaCl, 10 mM imidazole, 3 mM FC-12, pH 7.4) to Buffer B (25 mM Sodium Phosphate, 150 mM NaCl, 500 mM imidazole, 3 mM FC-12, pH 7.4).

100 μ L of the purified NfrB fraction was subsequently loaded on a size exclusion chromatography (SEC) column which contained superose 6 resin. According to the results in figure 2.9, fractions 27, 29, 33 and 35 from the SEC were collected and analyzed using 12% SDS-PAGE to detect the presence of NfrB. According to the results in figure 2.10, the SEC fractions 27 and 29 were collected and stored at -80 °C.

2.4.5 Amphiphilic polymer screen and Resolubilization in Cyclooctyl acrylamide (COA) – Acrylic Acid (AA) copolymer:

An initial amphiphilic polymer screen was carried out using Cyclooctyl Acrylamide – Acrylic Acid (Amphipol 18), Acrylic acid-co-styrene 6-50 (AASTY 6-50), The synthetic polymer SMALP 300 and Diisobutylene-maleic acid (DIBMA) Glucosamine as the candidates. 100 μ L of each polymer solution (2.5%) was mixed with 300 μ L of 40 mg/mL NfrB in MOPS buffer. This was incubated overnight at 4 °C while shaking. Each of them was centrifuged for 1 hour at 60,000 rcf and 4 °C. The supernatants were separated from the pellets. Into each of the supernatants, 40 μ L Methanol was added, vortexed to mix, 10 μ L chloroform was added, vortexed to mix, 30 μ L of MilliQ water was added, vortexed to mix and centrifuged for 3 minutes at 15,000 rcf. The upper aqueous layer was removed and 40 μ L of methanol was added. This was centrifuged

for 6 minutes at 17000 rcf. Each of the supernatants were separated from the pellets and analyzed by running on a 12% SDS gel.

According to the results COA-AA (Amphipol-18) was chosen as the suitable polymer for the resolubilization process.

For the resolubilization of NfrB in COA-AA copolymer, *E. coli* membrane fraction containing NfrB was solubilized in 2.5% COA-AA. This was centrifuged for 1 hour at 60000 rcf and 4 °C and subsequently incubated with 20 mL of Ni resin for 2 hours. Then NfrB was purified via FPLC on a HisTrap column via a linear gradient from Buffer A (25 mM Sodium Phosphate, 150 mM NaCl, 10 mM imidazole, pH 7.4) to Buffer B (25 mM Sodium Phosphate, 150 mM NaCl, 500 mM imidazole, pH 7.4). The resulting fractions which contained NfrB (confirmed by SDS-PAGE) were concentrated to a final protein concentration of 1 mg/mL.

Chapter 3: Structural characterization of the *in vitro* produced Nfr polysaccharide

3.1 Introduction

3.1.1 Polysaccharide biosynthetic mechanisms

There are at least three known biosynthetic mechanisms used by bacteria for polysaccharide formation; 1) The Wzx/Wzy-dependent flippase pathway; 2) The ATP binding cassette (ABC) transporter-dependent pathway; 3) The synthase-dependent pathway⁵. Each of these pathways (shown in Figure 3.1) use distinct mechanisms for polysaccharide biosynthesis and transport across the bacterial inner membrane.

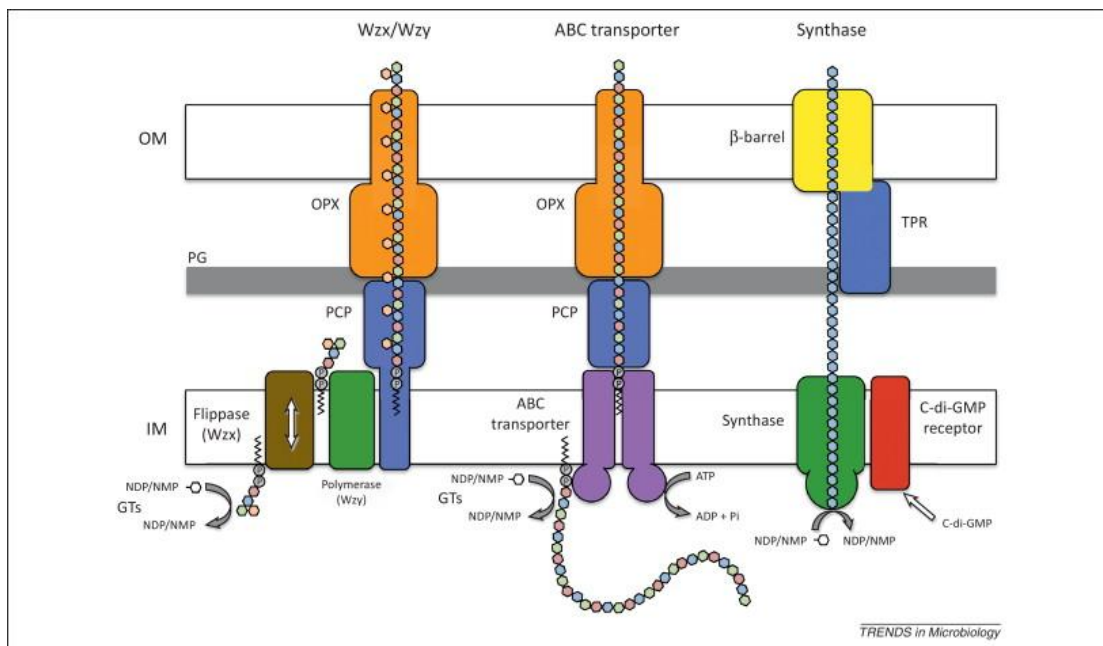


Figure 3.1. Biosynthetic mechanisms of polysaccharide synthesis and secretion², adapted from reference 47.

Assembly by the Wzx/Wzy-dependent pathway initiates in the cytoplasm with the generation of lipid-linked polysaccharide repeats⁴⁹. The first sugar unit gets added to the polyisoprenoid lipid carrier molecule undecaprenyl phosphate (UndP, C₅₅P) and this forms a Undecaprenyl Pyrophosphate (UndPP) linked sugar intermediate⁴⁹. After subsequent sugar unit additions by glycosyltransferase enzymes, the UndPP-linked repeat units are translocated from the inner membrane (IM) inner leaflet to the IM outer leaflet by the Wzx flippase⁴⁹. There, they are polymerized by Wzy⁴⁹. The O-antigen component of lipopolysaccharide (LPS) is made this way, and the final polymerized glycan is transferred onto the lipid A core oligosaccharide to form the final LPS molecule^{49,50}.

When considering the ABC transporter-dependent pathway, ABC transporters act as transporters or flippases, and utilize the energy of ATP hydrolysis to transport a plethora of compounds across cell membranes^{51,52}. Bacterial capsular polysaccharides (CPs) utilize this pathway in their synthesis⁵³. During this machinery, CPs get synthesized at the cytoplasmic face of the inner membrane via the collaborative actions of the glycosyltransferases⁵³. This is followed by it being exported across the cell membrane and translocated to the surface of the cell⁵³. The CPs that get synthesized in this method customarily contain a conserved reducing end glycolipid made out of phosphatidylglycerol as well as a poly-3-deoxy-D-manno-oct-2-ulosonic acid (Kdo) linker⁵³.

The synthase-dependent pathway consists of a complex between a membrane embedded GT and frequently a co-polymerase⁵. The synthase complex is responsible for both polymerization of the exopolysaccharide and translocation across the IM. For

biofilm exopolysaccharides specifically, c-di-GMP binding to the synthase complex has been shown to regulate the polymerization process. After translocation across the IM, polymer secretion is carried out by a protein with TPR domains and a β -barrel porin⁵. Usually, the proteins that are required for this pathway are encoded in a single operon⁵. Polysaccharides such as PNAG, cellulose and alginate utilize variants of this pathway⁵.

3.1.2 Nfr polysaccharide biosynthetic pathway

Nfr polysaccharide biosynthesis is believed to utilize the synthase-dependent pathway because it seems to be independent of a flippase activity for translocating repeat units. Synthase-dependent pathways are not believed to require a lipid-linked substrate for initiating the reaction.

However, there is some evidence that this may not be a general feature. For example, the *Salmonella enterica* O:54 biosynthesis pathway for *O*-antigen biosynthesis uses a synthase-dependent secretion mechanism that employs a undecaprenyl pyrophosphate linked disaccharide intermediate as an initiator substrate for the polymerization reaction⁵⁴. The homology between NfrB and the O:54 synthase is low, but it is possible that other synthase dependent pathways could employ lipid linked intermediates.

3.1.3 Importance of the Nfr polysaccharide structural characterization

Structural characterization of the polysaccharide synthesized by the Nfr system and the components of the Nfr polysaccharide secretion system itself will be critical for assessing the function of this pathway. According to literature and observations in this thesis, Nfr polysaccharide is a possible bacterial biofilm component. Since biofilm

formation is a strong virulence factor possessed by bacteria, gaining structural knowledge could lead us to novel targets that could be used in anti-biofilm treatment development⁵⁵.

3.2 Results and Discussion

3.2.1 In vitro synthesis and isolation of the Nfr polysaccharide

NfrB containing membrane fractions were incubated with c-di-GMP, WecB, MgCl₂ and UDP-GlcNAc for the synthesis of Nfr polysaccharide. As shown in Figure 3.2, a 20 mL reaction was set up containing 40 mg/mL of NfrB membrane fraction.

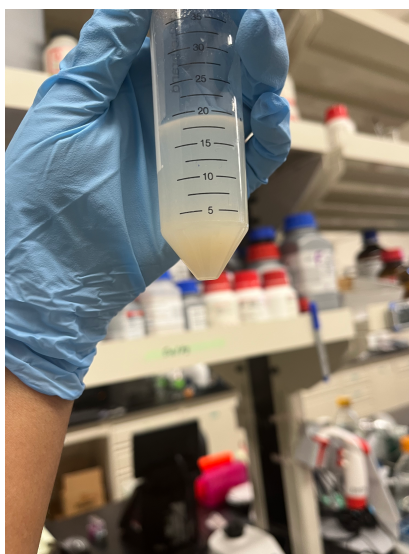


Figure 3.2. Starting reaction mixture containing c-di-GMP, WecB, MgCl₂ and UDP-GlcNAc for the synthesis of Nfr polysaccharide.

After incubation for 3 days at room temperature, followed by quenching of the enzymatic reaction through the addition of methanol. A two-phase Bligh-Dyer extraction was carried out to isolate the polysaccharide product in the insoluble

interface layer⁵⁶. Subsequently, the sample was treated with Proteinase K to remove any possible protein contaminants (Figure 3.3)



Figure 3.3. Reaction mixture post-MeOH quenching, Bligh-dyer extraction and Proteinase K treatment.

Finally, the sample was dried under vacuum for 6 hours. The resulting polysaccharide material, shown in figure 3.4, was insoluble in both water and organic solvents.



Figure 3.4. ManNAc polysaccharide product obtained from *in vitro* synthesis.

3.2.2 Characterization of the Nfr polysaccharide using MALDI-TOF

Initial characterization of the *in vitro* synthesized Nfr polysaccharide was carried out using matrix assisted laser desorption time of flight (MALDI-TOF) mass spectrometry (MS), using dihydroxybenzoic acid (DHB) as the matrix.

The resulting mass spectrum shows a series of peaks that are consistent with an increasing number of N-acetyl hexosamine repeats (Figure 3.5). Considering that a ManNAc residue has a mass of 221.09 Da, after the removal of water during polymerization, each ManNAc residue would be expected to add an additional 203.08 Da. Analysis of the MALDI-TOF MS results identified a series of peaks separated by 203 Da consistent with the expected poly-ManNAc polysaccharide. However, the m/z values observed do not correspond to the expected mass of a free reducing sugar polymer. Instead, the peaks suggest the polymer contains a yet to be identified aglycone, although the additional mass is consistent with that of UndPP-GlcNAc. An analysis of the peak intensities is consistent with a Gaussian distribution of ManNAc polymers ranging between ~12-50 ManNAc residues long and centered around 20. These length estimates were determined on the assumption that the poly-ManNAc polymer is attached to an UndPP-GlcNAc aglycone.

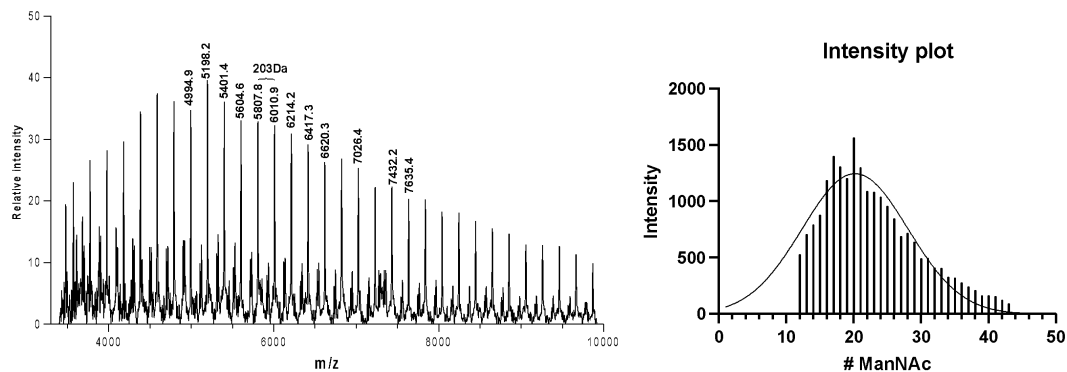


Figure 3.5. (a) MALDI-TOF mass spectrum (Relative intensity vs. m/z) of Nfr polysaccharide after being processed with RStudio and MALDIquant software (b) MALDI-TOF peak intensity vs. ManNAc residue number in a Gaussian distribution.

3.2.2 Characterization of the Nfr polysaccharide using solid-state ^{13}C NMR

Since the *in vitro* synthesized Nfr polysaccharide was insoluble in both aqueous and organic solvents, characterization was instead carried out using magic angle spinning (MAS) cross polarization (CP) solid state ^{13}C NMR spectroscopy. The Nfr polysaccharide spectra was compared to that of crystalline β -ManNAc monosaccharide.

As seen on Figure 3.3, the major peaks are most consistent with a ManNAc homopolymer connected through β -(1 $\rightarrow$ 4)glycosidic bonds, although an alternative β -(1 $\rightarrow$ 6)-linked homopolymer cannot be conclusively ruled out. Observing the differences and similarities between the two spectra, one thing that can be observed is that the peaks corresponding to C2, C3, C5, C7 and C8 have not significantly shifted. When coming down from the monomer spectrum to the polymer spectrum, they seem to only have broadened and shortened, which can be expected as a result of the increased size of the polymeric material. However, the peak corresponding to C1 is

shifted downfield in the polymer spectrum. In addition to that, the peak corresponding to either C4 or C6 is shifted as well. Due to the low resolution of the spectra, it is not possible to definitively determine which of the two carbon peaks has shifted. A downfield shift of ^{13}C peaks is indicative of a change in chemical environments around the corresponding carbon atoms. In this instance, the indicated chemical environment change results from glycosidic bond formation either between C1 and C4 atoms or C1 and C6 atoms. Hence, from these observations alone, it could be said that the glycosidic bonds between ManNAc residues is either β -(1 $\rightarrow$ 4) or β -(1 $\rightarrow$ 6).

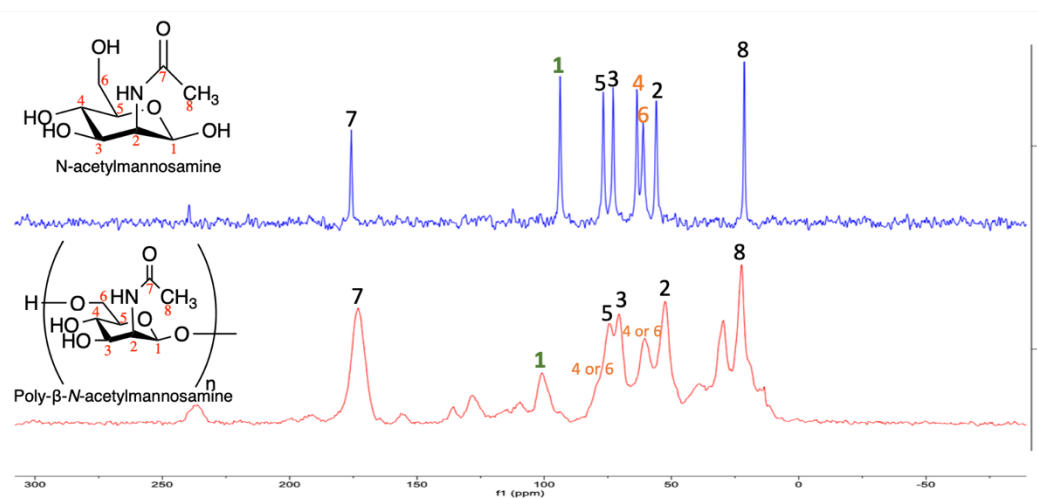


Figure 3.6. solid-state ^{13}C NMR spectra. Top spectrum is for free ManNAc. Bottom spectrum is for the putative ManNAc polymer.

It is commonly observed that glycosidic bond formation tends to lead to the broadening of the peaks corresponding to the carbon atoms participating in the glycosidic bond, while the relative peak area for the other peaks remains largely unchanged. The observed peak at 61 ppm is thus likely to correspond to C6 of the poly-ManNAc

polysaccharide, and the peak corresponding to C4 has shifted and broadened upon glycosidic bond formation.

Thus, the NMR spectrum of the polysaccharide is most consistent with a β -(1 $\rightarrow$ 4)-linked poly-ManNAc, although a β -(1 $\rightarrow$ 6)-linked poly-ManNAc cannot be definitively ruled out (Figure 3.4). Additional NMR experiments using C6- ^{13}C modified ManNAc polysaccharides could be used to conclusively distinguish between these two possibilities.

In addition to peaks corresponding to the poly-ManNAc polymer, there are additional peaks present in the spectra that are consistent with those expected for a UndPP lipid. Specifically, the peaks at ~ 135 and ~ 128 ppm are consistent with an alkene functional group and the additional peaks between 20-40 ppm are consistent with UndPP alkyl carbons. UndPP linked intermediates are common in bacterial polysaccharide biosynthetic pathways but have not been implicated in any of the characterized synthase dependent exopolysaccharide biosynthesis pathways studied to date. Thus, additional characterization is required to establish the presence of a UndPP linked product and to distinguish between the two possible glycosidic linkage positions in this polymer.

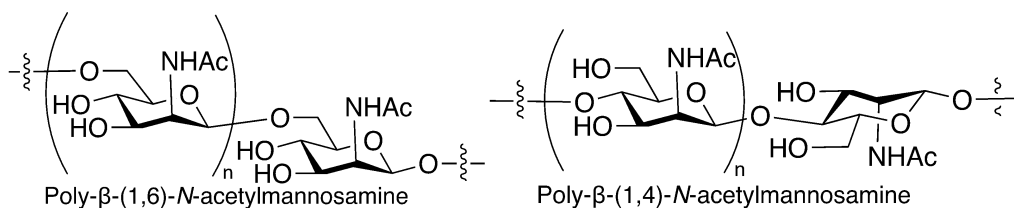


Figure 3.7. Possible Nfr polysaccharide structures.

3.3 Conclusions

In-vitro synthesis of Nfr polysaccharide requires UDP-GlcNAc, WecB, c-di-GMP and MgCl₂. The Nfr polysaccharide structure is consistent with a ManNAc homopolymer connected through β -(1→4) or β -(1→6) glycosidic linkages although there is a higher chance that the bonds are β -(1→4). Additionally, this polymer is most likely lipid linked. The mechanism by which this synthesis occurs is most likely, a hybrid of the synthase-dependent pathway and the Wzx/Wz-dependent flippase pathway.

3.4 Experimental Details

3.4.1 Synthesis and ¹³C solid state NMR analysis of Nfr polysaccharide

To a 20 mL reaction mixture, MOPS, 1 mM MgCl₂, 10 mM UDP-GlcNAc, 0.5 μ M WecB, 2 μ M c-di-GMP, 200 μ M WecB and 40 mg/mL NfrB were added. The mixture was incubated for 3 days at room temperature. An equal volume of cold ethanol was then added to quench the reaction and centrifuged for 20 minutes at 3220 rcf at 4 °C. The resulting solid was stored at -20 °C overnight. Afterwards, the solid was redissolved in 10 mL water and 10 mL MeOH. This was centrifuged for 20 minutes at 3200 rcf at 4 °C. The resulting pellet was collected and resuspended in water. 10% triton X-100 was added into this, and spin centrifuged for 10 minutes at 3200 rcf at 4 °C. The resulting pellet was washed with 10 mL of water 3 times. Next, Bligh/Dyer extraction of total lipids was carried out as follows. 3 mL of 3:1 Methanol: Chloroform was added into the pellet and mixed on nutator for 20 minutes. To this, 1 mL chloroform and 1.8 mL water were added, shaken and centrifuged at 17000 xg. The insoluble disc that formed between the upper aqueous layer and lower organic layer was collected. To this,

5 mL MOPS buffer and 25 μ L Proteinase K (10 mg/mL) were added. The mixture was heated in a water bath for 1 hour at 42 °C. It was then washed twice with pure water for a total of 10 minutes, and pelleted by centrifugation after each wash. The resulting solid was suspended in a minimal volume of MiliQ water and dried using a SpeedVac for 6 hours, to give 29.47 mg of solid product.

This was subjected to Magic Angle Spinning (MAS) – Cross Polarization (CP) solid state ^{13}C NMR analyses with the assistance of Dr. Bin Chen and Dr. Fu Chen. The 29.47 mg solid product was crushed using a mortar and pestle and the resulting powder was packed into a tube. This was followed by MAS-CP solid state ^{13}C NMR analysis. *N*-acetylmannosamine was used as a control.

3.4.2 MALDI-TOF structural analysis of Nfr polysaccharide

In order to perform MALDI-TOF mass spectrometry, the sample preparation was carried out. Firstly, the 29.47 mg of polysaccharide sample was resuspended in deionized water 50 μ L. This suspension was sonicated for 15 minutes to break the sample down further. After that sample loading onto the plate was carried out using dihydroxybenzoic acid (DHB) as the matrix. A MALDI-MS 2 mm 384-well stainless-steel sample plate was utilized and DHB 1 μ L was added onto 3 wells each. It was left to dry at room temperature for 5 minutes and 1 μ L of the sample was added on top of each dried matrix drop. After 5 minutes of drying, another 1 μ L of the DHB was added on as a third layer. MALDI-TOF mass spectrometry was performed on a Bruker Autoflex Speed spectrometer and each sample was measured in triplicates.

Chapter 4: Development of biological tools to detect the Nfr polysaccharide in bacterial biofilms.

4.1 Introduction

4.1.1 Importance of biofilm exopolysaccharide detection

As the most abundant component in bacterial biofilms, exopolysaccharides carry a high level of significance⁵⁷. Most of the physicochemical properties of these biopolymers are closely related to the three-dimensional structure of the biopolymer network it resides in⁵⁷.

Detection of exopolysaccharides in bacterial biofilms can not only be a convenient method of characterizing biofilms, but these methods can also aid in the development of anti-biofilm therapeutics.

4.1.2 Detection of exopolysaccharides in bacterial biofilms

Surprisingly, despite the ubiquitous nature of exopolysaccharides, there seem to be relatively low number of proteins that have the ability to recognize these biopolymers⁵⁸. When considering the cellulose biosynthetic pathway, BcsZ is a periplasmic enzyme that contains β -endo-1,4-glucanase activity and it is responsible for degradation of cellulose accumulating in the periplasm⁵⁹. With regards to the PNAG biosynthetic machinery, Dispersin B (DspB) is one of the only two enzymes that has the ability to act specifically on the β -1,6-GlcNAc structure⁵⁸.

Researchers have utilized this unique specificity of the PNAG specific glycosyl hydrolase DspB, to develop a macromolecular fluorescent PNAG binding probe⁵⁸. An

active site mutant of DspB has fused to a fluorescent protein, resulting in a probe that has the ability to only bind to PNAG, yet not hydrolyze it⁵⁸. Due to the specificity of DspB, this probe has been used to study PNAG in the context of growing biofilms where the probe sequesters in areas that contain high levels of PNAG⁵⁸.

4.1.3 Periplasmic protein YbcH and its utilization in developing an exopolysaccharide tool

YbcH is a periplasmic protein, encoded in the same operon as NfrA and NfrB³³. It belongs to the DUF4434 family, which means that it is a domain with an unknown function. According to literature, YbcH is not essential for Nfr polysaccharide synthesis and secretion³³.



Figure 4.1. Predicted AlphaFold structure of YbcH protein.

The AlphaFold predicted structure of YbcH (Figure 4.1) contains a significant structural similarity to the starch hydrolyzing enzyme beta amylase. Beta amylase belongs to glycosyl hydrolase 14 (GH14) family and exhibits a substrate inverting mechanism for hydrolysis³⁹. Catalytic machinery and molecular mechanism are

features that are usually conserved for the vast majority of the glycosidase families. Based on this knowledge, an assumption was made during this study that YbcH could contain potentially similar glycosyl hydrolase activity. Under this assumption, experiments have been carried out to develop this protein into a potential Nfr polysaccharide binding and detection tool.

4.2 Results and Discussion

4.2.1 Expression and purification of recombinant YbcH in *E.coli* BL21(DE3) cells

The gene encoding YbcH from *Escherichia coli* containing a *N*-terminal hexahistidine tag (YbcH-His₆) was cloned into a pET28 vector and expressed in BL21(DE3) cells. YbcH was purified via fast Protein Liquid Chromatography (FPLC) on a HisTrap column and buffer exchanged, resulting in a pure and catalytically active approximately 33 kDa YbcH protein (Figure 4.2).

A catalytically inactive YbcH variant (E245A) with a fluorescent mCherry tag was also prepared in the lab by Sureshee Liyanaarachchi. This had been expressed and purified using similar methods as YbcH. The YbcH-mCherry conjugate has an excitation wavelength of 587 nm and an emission wavelength of 610 nm and can be used as a possible Nfr polysaccharide detection tool.

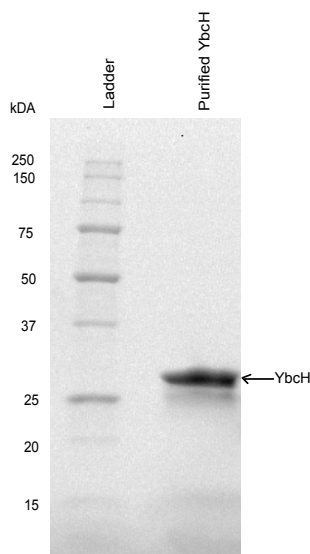


Figure 4.2. SDS-PAGE gel showing HisTrap column purified YbcH which is approximately 32.8 kDa.

4.2.2 Fluorescence-based dot blot assay for Nfr polysaccharide identification

MALDI-TOF analysis of the *E. coli* membrane fraction containing NfrB showed the presence of Nfr polysaccharide. As a result, it was hypothesized that some amount of Nfr polysaccharide is present in the membrane fraction as a result of NfrB activity in the overproducing strain. To identify the presence of Nfr polysaccharide in *E. coli* membranes, we developed a fluorescent-based dot blot assay using YbcH-mCherry as a detection reagent. NfrB containing membrane fractions were compared to empty vector membrane fractions. Both membrane fractions were additionally incubated in the presence and absence of active YbcH before spotting on nitrocellulose and detection with YbcH-mCherry as seen in Figure 4.3.

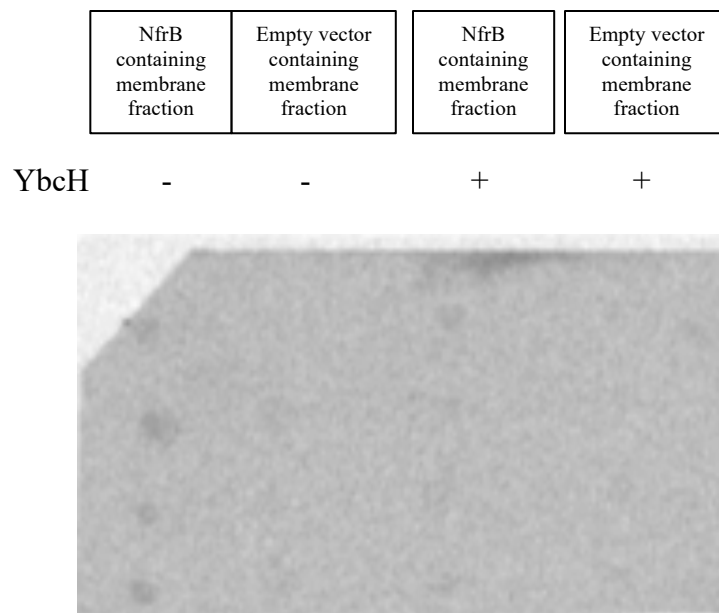


Figure 4.3. The result of the fluorescence-based dot blot assay.

YbcH-mCherry labeling was observed for the NfrB membrane fractions, but not in empty vector control membranes. Moreover, treatment of the NfrB membranes with wild type YbcH prior to analysis resulted in a decrease in YbcH-mCherry binding. These results indicate that Nfr polysaccharide is present in the *E. coli* membrane fraction containing NfrB. Additionally, the results also indicate that YbcH has ability to hydrolyze Nfr polysaccharide. Thus, wild type YbcH is likely an Nfr polysaccharide hydrolase enzyme and the YbcH-mCherry conjugate can serve as a Nfr polysaccharide binding and detection tool.

4.3 Conclusions

Periplasmic protein YbcH likely contains the ability to bind and hydrolyze the Nfr polysaccharide. By preparing a catalytically inactive YbcH mutant fused with a

monomeric mCherry fluorescent protein, this probe can be used as a Nfr polysaccharide binding and detection tool. In addition, Nfr polysaccharide was observed in the membrane fraction of NfrB overproducing cells but absent in empty vector control membranes.

4.4 Experimental Details

4.4.1 Expression and purification of YbcH protein

A DNA sequence for the expression of *E. coli* YbcH containing a *N*-terminal hexahistidine (His₆-YbcH) was synthesized, codon optimized for expression in *E. coli* and cloned into a pET28 vector to give a pET28:YbcH plasmid construct. BL21(DE3) cells transformed with this plasmid were grown overnight at 37 °C with shaking in 50 mL of LB broth containing 50 µg/mL kanamycin. 5 mL of overnight culture was transferred to 500 mL of fresh LB broth containing 100 µg/mL kanamycin and grown to OD₆₀₀ of 0.4-0.6, then induced with IPTG to a final concentration of 1 mM. The cultures were then grown overnight at 17 °C with shaking. At this point the cell pellet was harvested by centrifugation for 20 minutes at 5500 rcf at 4 °C. Cell pellets could be stored at -20 °C before lysis. The cell pellet was then resuspended to 10 mL/1 g in wash buffer (25 mM Tris HCl, 300 mM NaCl, 5% glycerol, 10 mM imidazole, pH 7.4), and lysed by sonication (30% amplitude, 1 second on, 3 seconds off for a total of 2.30 minutes on. This was repeated 3 times). After sonication, the cell debris was pelleted 20 minutes at 20000 xg at 4 °C. The lysate was then purified via FPLC on a HisTrap column using a linear gradient from Buffer A (25 mM Tris HCl, 300 mM NaCl, 5% glycerol, 10 mM imidazole, pH 7.4) to Buffer B (25 mM Tris HCl, 300 mM NaCl, 5%

glycerol, 500 mM imidazole, pH 7.4). The purified protein was then buffer exchanged into MOPS. The resulting 32.8 kDa protein was then concentrated to 300 μ M and stored at -80 °C.

4.4.2 Fluorescence-based dot blot assay to test YbcH hydrolytic activity

4 test reactions were carried out as follows:

1. *E.coli* membrane fraction containing NfrB (5.5 mg/mL) 50 μ L + 30 μ M YbcH 5 μ L
2. *E.coli* membrane fraction containing NfrB (5.5 mg/mL) 55 μ L (Untreated with YbcH)
3. Empty vector *E.coli* membrane fraction 50 μ L + 30 μ M YbcH 5 μ L
4. Empty vector *E.coli* membrane fraction 50 μ L (Untreated with YbcH)

0.5 μ L of each of these reactions were dotted on a nitrocellulose filter paper. This was washed with 10 mL of the blocking agent Phosphate Buffered Saline (PBST) + 2% Bovine Serum Albumin (BSA). 3 5-min washing steps were subsequently carried out using PBST. Afterwards, YbcH-mCherry conjugate 10 μ L was added. Fluorescence emission was visualized. Resulting image has been shown in figure 4.3.

Chapter 5: Summary and future directions

Bacteria possess the ability to make biofilms, which are structured communities of bacterial cells firmly attached to a surface and embedded in a matrix composed of EPS⁶⁰. They are considered a strong virulence factor and are known to mediate over 80% of chronic infectious diseases⁴. The biofilm EPS contains exopolysaccharides, proteins, eDNA and lipids among other minor components. Out of these, exopolysaccharides play a vital role in biofilm formation and maintenance.

Escherichia coli K-12 is known to produce mainly 2 exopolysaccharides namely, phospho-ethanolamine modified cellulose (PEC) and PNAG. However, a more recent discovery led to a novel c-di-GMP regulated polysaccharide synthase that was previously only known as necessary for N4 phage infection^{33,34}. Moreover, it was revealed that this polysaccharide has the ability to inhibit bacterial motility, which led us to the hypothesis that this novel Nfr polysaccharide could be playing a role in bacterial biofilm formation.

In *E.coli*, NfrB is encoded in an operon with NfrA and YbcH³³. Together, these three proteins show the key characteristics of a Gram-negative polysaccharide synthesis and secretion system.

During our studies, we were able to develop a system that allows successful overexpression of recombinant NfrB protein in the membrane fraction of *E. coli*. A HPLC-based activity assay was developed to measure the activity of NfrB by monitoring the production of UDP.

Using active NfrB protein, milligram quantities of the Nfr polysaccharide was synthesized *in vitro*. Subsequent structural characterization of the polysaccharide

utilizing solid-state ^{13}C NMR and MALDI-TOF mass spectrometry suggests that the Nfr polysaccharide is a lipid-bound ManNAc homopolymer. The NMR are most consistent with a poly- β -(1 $\rightarrow$ 4)-*N*-acetylmannosamine homopolymer; however, additional MALDI-TOF and NMR analysis is required to confirm this structure. Specifically, solid state ^{13}C NMR on Nfr polysaccharide prepared using C6- ^{13}C labeled UDP-GlcNAc in the presence of NfrB, WecB and c-di-GMP, could be used to differentiate between β -(1 $\rightarrow$ 4)- and β -(1 $\rightarrow$ 6)-linked ManNAc homopolymers by looking at the chemical shift of C6 in the polysaccharide.

In addition, my studies revealed that the NfrB GT enzyme activity depends on c-di-GMP and WecB. Moreover, our experiments revealed that, in order to isolate the NfrB protein from its native membrane environment, FC-12 is suitable for detergent resolubilization and COA-AA is suitable for lipid nanodisc formation.

However, it was also revealed that NfrB isolation from its native lipid membrane causes the protein to lose its GT enzyme activity. We hypothesize that this could be due to the loss of PGT enzyme activity that may be present in the membrane and is required for synthesis of UndPP-GlcNAc. This needs to be extensively tested using authentic undecaprenyl pyrophosphate N-acetylglucosamine (Und-PP-GlcNAc) as a possible initiator substrate.

As shown in figure 5.1, WecA, a bacterial PGT is responsible for the formation of UndPP-GlcNAc in the bacterial inner membrane. Certain experimental results observed thus far, such as the MALDI-TOF data, point in the direction of this UndPP-GlcNAc being the possible initiator for Nfr polysaccharide biosynthesis. Polyprenol pyrophosphate linked intermediates are common in bacterial polysaccharide bio

synthetic pathways, but they have not been implicated in synthase dependent exopolysaccharide bio synthetic pathways. Therefore, this needs additional experimental support. These additional experiments have been planned to be carried out in collaboration with Professor Jerry Troutman from University of North Carolina at Charlotte.

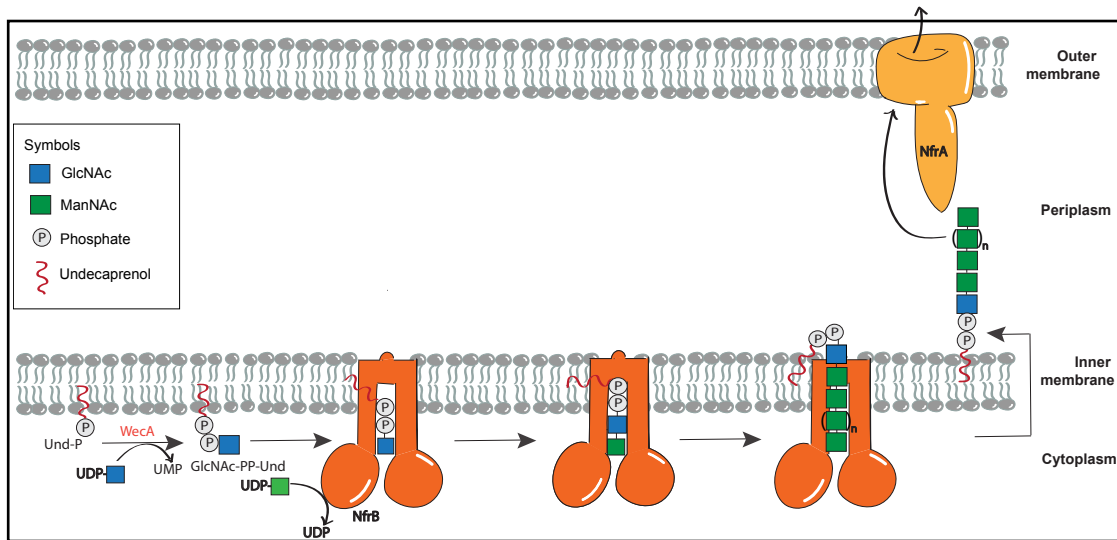


Figure 5.1. A possible Nfr polysaccharide synthesis mechanism involving UndPP-GlcNAc as the possible initiator substrate.

As another part of this study, YbcH was developed as a Nfr polysaccharide binding and detection tool. This was done by attaching a fluorescent tag to inactive YbcH protein. Since the results that has been gathered so far, to test the affinity between YbcH and Nfr polysaccharide are at the preliminary stages, more testing needs to be carried out. A fluorescent based dot assay has been developed for this purpose.

In addition to YbcH, previous research indicates that N4 virion protein gp65 directly interacts with NfrA during viral adsorption⁴¹. More recent research suggests that the novel Nfr polysaccharide is used by the phage N4 as an initial receptor for adsorption¹²

(Figure 5.2). Based on this, N4 gp65 could be tested as a potential Nfr polysaccharide binding protein. An initial pull-down assay could be carried out to assess the affinity between the two components and depending on the results, N4 gp65 could be developed as a potential Nfr polymer binding tool.

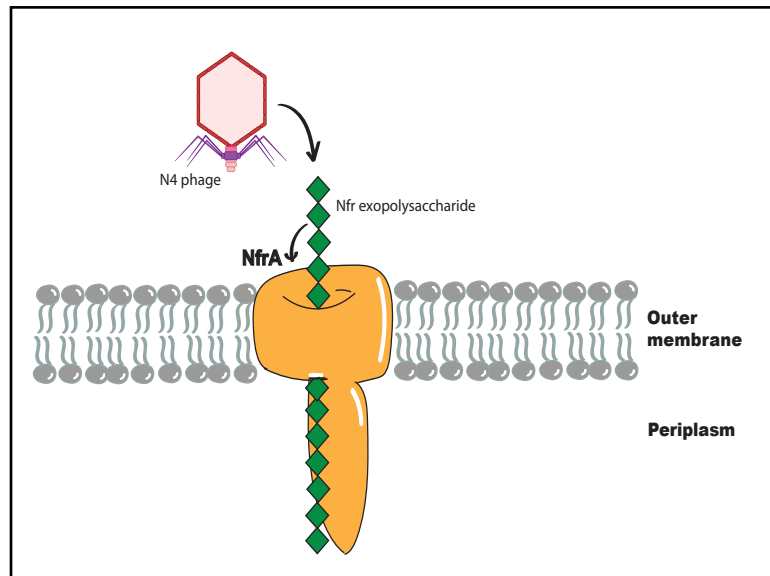


Figure 5.2. The interaction between N4 virion and *E.coli* could be potentially be, initially between gp65 and the Nfr polysaccharide and subsequently between gp65 and NfrA.

Evaluating the localization of Nfr polysaccharide and the functions of the individual components of the Nfr system in *E. coli* is another possible future direction this study could take. In order to do this, preparation of *E.coli* Nfr pathway overproducing and knockout strains for NfrA, NfrB, YbcH and WecA (Figure 5.3) need to be carried out.

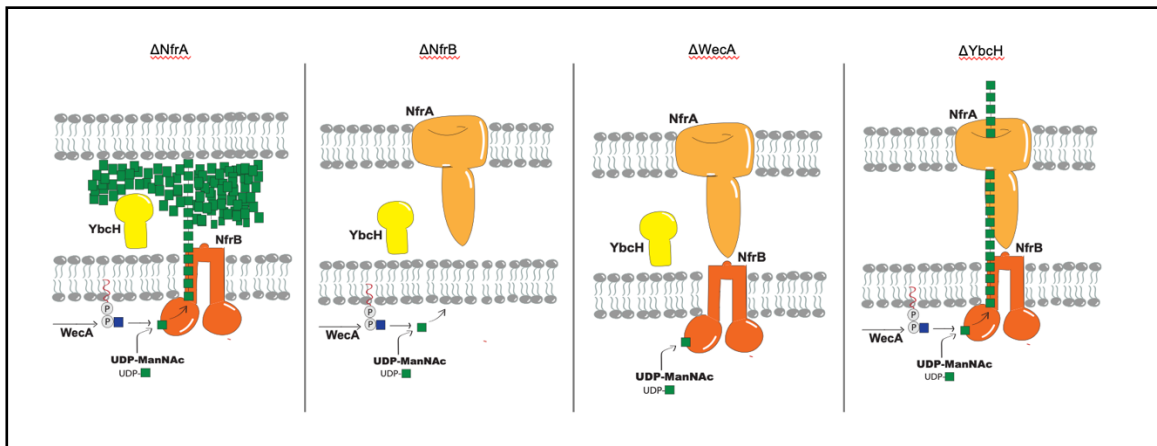


Figure 5.3. Graphical representation of the possible outcomes for the mutant strains Δ NfrA, Δ NfrB, Δ WecA and Δ YbcH. Δ NfrA could possibly cause an accumulation of the Nfr polysaccharide in the periplasm. For both Δ NfrB and Δ WecB, a loss of Nfr polysaccharide production altogether could be expected. In the case of Δ YbcH, since there is evidence that YbcH is not required for the synthesis or secretion of Nfr polysaccharide, it's not possible to predict what the outcome would be.

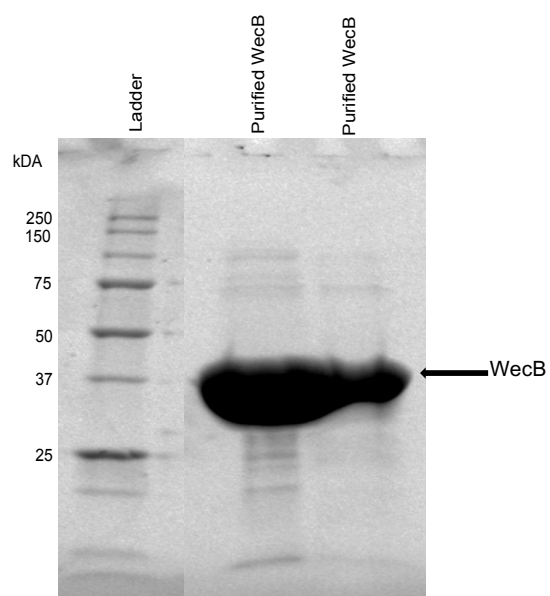
Subsequent microscopic studies could be performed to evaluate the localization of the Nfr polysaccharide in *E.coli* and its possible biological function can be assessed by comparing Nfr polysaccharide production in planktonic vs. biofilm *E. coli* cells.

Appendix

Materials and methods

Expression and purification WecB protein

A DNA sequence for the expression of *E. coli* WecB containing a *N*-terminal hexahistidine (His₆-WecB) was synthesized, codon optimized for expression in *E. coli* and cloned into a pET28 vector to give a pET28:WecB plasmid construct. BL21(DE3) cells transformed with this plasmid were grown overnight at 37 °C with shaking in 50 mL of LB broth containing 50 µg/mL kanamycin. 5 mL of overnight culture was transferred to 500 mL of fresh LB broth containing 100 µg/mL kanamycin and grown to OD₆₀₀ of 0.4-0.6, then induced with IPTG to a final concentration of 1 mM. The cultures were then grown overnight at 17 °C with shaking. At this point the cell pellet was harvested by centrifugation for 20 minutes at 5500 rcf at 4 °C. Cell pellets could be stored at -20 °C before lysis. The cell pellet was then resuspended to 10 mL/1 g in wash buffer (25 mM Tris HCl, 300 mM NaCl, 5% glycerol, 10 mM imidazole, pH 7.4), and lysed by sonication (30% amplitude, 1 second on, 3 seconds off for a total of 2.30 minutes on. This was repeated 3 times). After sonication, the cell debris was pelleted 20 minutes at 20000 xg at 4 °C. The lysate was then purified via FPLC on a HisTrap column using a linear gradient from Buffer A (25 mM Tris HCl, 300 mM NaCl, 5% glycerol, 10 mM imidazole, pH 7.4) to Buffer B (25 mM Tris HCl, 300 mM NaCl, 5% glycerol, 500 mM imidazole, pH 7.4). The purified protein was then buffer exchanged into MOPS. The resulting 44.5 kDa protein was then concentrated to 200 µM and stored at -80 °C.



Supplementary Figure 1.1. SDS-PAGE gel showing HisTrap column purified WecB which is approximately 44.5 kDa.

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