ENGINEERING ESCHERICHIA COLI FOR NOVEL VESICLE VACCINE METHODS

by Christine Elizabeth Endicott

ENGINEERING *ESCHERICHIA COLI* FOR NOVEL VESICLE VACCINE METHODS

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Christine Elizabeth Endicott

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ABSTRACT

Vaccines are one of the most economically effective courses of preventative medicine and their efficacy has been proven in the eradication of many deadly, infectious diseases [3]. Despite their success in developed nations, the World Health Organization estimates that 2.5 million children worldwide still die of vaccine-preventable diseases and of these children 70% are in the world's poorest nations. Among the many vaccine strategies, glycoconjugate vaccines are an effective method, however their high cost of development and manufacture makes them inaccessible to developing nations. Thus, the main goal of this research is to employ bacteria for the development of low-cost glycoconjugate vesicle vaccines. A vesicle vaccine takes advantage of the inherent immunostimulatory properties of the components naturally found in the outer membrane which are included when parts of the outer membrane bleb off as outer membrane vesicles, or OMVs. We hypothesize that glycoconjugate vesicle vaccines will address many of the current challenges surrounding glycoconjugate vaccines and make them attainable worldwide. This research has shown that we can produce two types of glycoconjugate vaccines. One variation is the conjugation of heterologous bacterial polysaccharides to lipid A, an adjuvant yet toxic component of lipopolysaccharide, or LPS, which is a major component of OMVs. The second variation is to utilize a conjugating enzyme, an oligosaccharyltransferase (OST), from the bacterium *Camphylobacter jejuni* to attach the bacterial polysaccharide to an outer membrane protein, which will bleb off with the outer membrane vesicles. Both techniques utilize the stability and delivery benefits of bacterial outer membrane vesicles. This research has the potential to offer a stable, immunogenic, non-toxic, all-in-one vaccine and delivery system that may offer

protection against diverse infectious diseases that is inexpensive and accessible to developing nations.

BIOGRAPHICAL SKETCH

Christine Elizabeth Endicott was born in Burlington, Vermont on March 15, 1986. She grew up in Essex Junction, Vermont with her parents and younger brother Jeffrey. In June 2004, she graduated third in her class from Essex High School in Essex Junction, Vermont. In August of 2004 she began studies at the University of Connecticut in the Honors Program to study chemical engineering. As an undergraduate, Christine completed two internships. During the summer of 2006 she was a customer quality engineering intern for IBM Microelectronics in Essex Junction, Vermont. In the summer of 2007 she was a systems engineer at UTC Power in South Windsor, Connecticut where she analyzed hydrogen fuel cell systems. While at the University of Connecticut, she performed undergraduate research in *Bacillus anthracis* mitigation with Professor Ranjan Srivastava. In May 2008, Christine graduate summa cum laude from the University of Connecticut with a BS in chemical engineering. In August of the same year, she began her graduate studies in chemical engineering at Cornell University in Ithaca, New York with Professor Matthew DeLisa. In August of 2010, she received her MS in chemical engineering. In September, she started her career as a process development engineer at Regeneron Pharmaceuticals in Tarrytown, New York.

to my Parents, Jeffrey, and James

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CHAPTER 1: INTRODUCTION

Conjugate Vaccines & Immune Response

The clinical goal of vaccines is to induce an immune response that will lead to the continuous production of antibodies against an infection by introducing all or part of an infection carrier to a host. This is achieved by stimulating both lymphocytes from bone marrow (B cells) and lymphocytes that matured in the thymus (T cells). Our focus is on glycoconjugate vaccines, which are a bipartite structure consisting of an infectious bacterial antigen conjugated to an adjuvant carrier. The purpose of the adjuvant is to enhance the immune response while the antigen is the component specific to a pathogen that will be recognized by the immune system should the infection be encountered post immunization. Polysaccharides are often very specific to bacterial and viral species thus they make good antigen candidates. Adjuvants are immunostimulatory specifically in activating both B cell and T cell response. Toxins from bacteria are often used as adjuvants because they often have properties that the immune systems responds to in a way that gives rise to immunological memory.

Proteins are known to elicit a T cell dependent response directly, and are used in current glycoconjugate vaccine technologies [5]. When both a protein and polysaccharide are conjugated together and introduced to a host, the immune system recognizes the foreign body and presents the protein to T cells, which then become active. The B cells immediately produce short-term antibodies against the polysaccharide as they would have if there been no protein conjugation. The T cell involvement leads to the development of memory B cells that will produce active serum antibodies against the bacteria-specific polysaccharide, which are necessary should that bacterial infection be encountered in the future [5, 6]. An indirect way to activate the immune system is to use an adjuvant that induces macrophage activation.

Once a macrophage digests a foreign toxin, antigen-presenting cells then take those components that ultimately lead to the stimulation of T cells [7].

A successful example of a glycoconjugate vaccine is the *Haemophilus influenzae* type b, or Hib, vaccine [5, 8, 9]. The Hib vaccine uses a capsular polysaccharide from the surface of the bacterium, which is chemically cross-linked to a protein. Typically, the protein is a tetanus toxoid, diphtheria toxoid, diphtheria protein, or a protein complex from *Neisseria meningitidis,* all of which are highly immunogenic, or stimulate an active immune response [8]. This vaccine has drastically reduced the incidence of this bacterial infection in developed countries [10].

Current Methods for Conjugate Vaccine Production

Unfortunately, current methods for producing conjugate vaccines are inefficient, very expensive, introduce toxic chemicals and allergens, and require the handling of dangerous pathogens to grow and isolate the bacterial polysaccharides [11, 12]. There have been methods presented for synthetic formation of Hib vaccines via chemical synthesis of the polysaccharide antigen however these methods run into similar issues with the introduction of toxins used to modify the proteins and sugars for cross-linking [9, 11]. One way to conjugate a polysaccharide to a protein, according to current methods to produce the Hib vaccine, involves the use of cyanogen bromide, which modifies protein structures for conjugation, and is extremely poisonous [8]. In addition to the chemicals used, the production of conjugate vaccines is very lengthy requiring at least eight purification and modification steps as shown in Figure 1. Lengthy procedures and the use of chemicals inevitably drive up the costs of production.

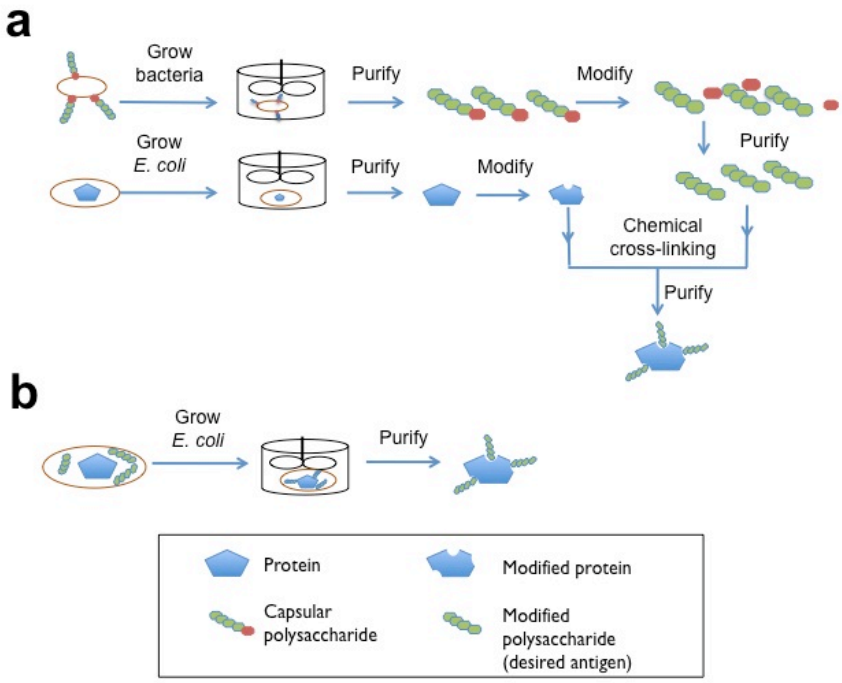


Figure 1: Current methods of conjugate vaccine production. (a) demonstrates the current methods for producing the Hib vaccine. A pathogen must be grown so that the capsular polysaccharide may be isolated and modified for cross-linking. The protein antigen is produced in a separate culture and is also isolated and modified before chemical cross-linking. (b) is the current technology being used by GlycoVaxyn where all steps are done *in vivo* reducing the processing to a single culture and purification step.

Lipopolysaccharide

Lipopolysaccharide (LPS) is a structure that is attached to the outer leaflet of the outer membrane in gram-negative bacteria. LPS is comprised of lipid A, core sugars, and O-antigens as shown in Figure 2. Lipid A anchors the molecule by extending into the outer membrane; attached to lipid A are the core sugars. The core sugars are comprised of 3-deoxy-D-manno-octulosonic acid (Kdo) sugars which are considered to be the minimal sugar necessary in LPS for *E. coli* to be viable [13, 14]. *O*-antigen polysaccharides are the outermost part of LPS, connected to the core sugars [15]. Lipid A is an endotoxin, or bacteria-associated toxin, that causes septic shock which can lead to death [15, 16]. The immune system is so responsive to lipid A that the molecule induces the immune system to overproduce the molecules that are associated with the inflammatory response, which causes the sepsis [7]. Lipid A is mostly conserved among bacterial species, whereas the sugars become increasingly varied moving away from lipid A [17]. *O*-antigen polysaccharides are the most diverse component of LPS among bacteria and are specific to a bacterial species.

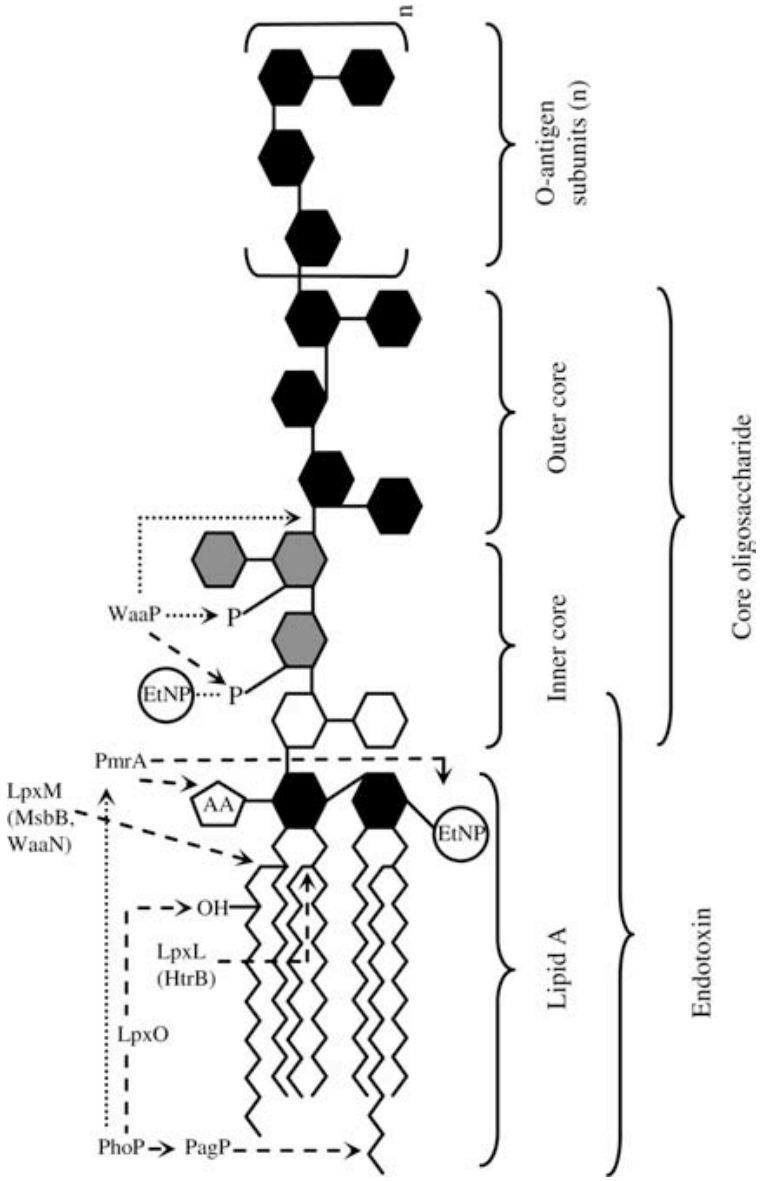


Figure 2: Structure of LPS. Taken from Nagy et al [2]. Note the three major components: lipid A or endotoxin, the core sugars, and the *O*-antigen.

Bacterial *O*-Antigens

O-antigen construction starts on the inner leaflet of the inner membrane in the cytoplasm, a series of glycosyltransferases attach many sugar subunits, which are to be flipped to the periplasmic side by Wzx flippase [17]. The *O*-antigen ligase, which attaches an *O*-antigen molecule to the core sugars, is catalyzed by WaaL [18]. *O*-antigens are common in pathogenic gram-negative bacteria, and due to their specificity to bacterial species they important molecules in designing safe, effective vaccines. Because *O*-antigens are so unique to a species, the immune system is able to create serum antibodies that are very specific will be able to recognize the pathogen based on its polysaccharides in the future. Additionally, a great advantage of using *O*-antigens for vaccine development and implementation is that the genes involved in the synthesis exist in clusters in the genome and thus can be isolated and cloned, eliminating the need to handle pathogens. This increases the safety for all those who are involved in the research and production of these vaccines.

Unfortunately, *O*-antigens alone cannot be used as vaccines as they elicit a T cell independent response, which means T cells are not stimulated [5]. B cells initially are stimulated to create antibodies against the *O*-antigen, but they are not continually produced in the serum and thus the immune system would not recognize these molecules if they were to be encountered again. Memory B cells, which continually produce antibodies, are not created without T cell activation and hence there is no protection upon repeated exposure to the same pathogen. Use of a polysaccharide-only vaccine is also problematic and limited because of various immune disorders and children are not able to produce antibodies against polysaccharides until they are around 5 years of age. Because younger children are more susceptible to disease, a polysaccharide-only vaccine would not be very useful.

A new adjuvant 3-*O*-desacyl-4'-monophosphoryl lipid A (MPL) has been FDA-approved for vaccines [19]. This structure is of interest to our work as this molecule is a similar structure to the lipid A found in most gram-negative bacteria such as *E. coli*. Studies have shown that MPL is involved in cellular immunity, recruiting macrophages to break down the components of the foreign body [4]. It is believed that this adjuvant is involved in activating cytokine cascades and antigen presenting cells [20]. The antigen presenting cells then present the antigens to the T cells necessary for immunologic memory and MPL has actually been shown to inactivate suppressor T cells [21]. MPL is derived from the *Salmonella minnesota* lipid A where it is purified then chemically modified [20]. The first modification was discovered by Ribi et al [22]. They found that the removal of a phosphate group from the lipid A disaccharide reduced the toxicity of lipid A at least 100 fold. However, the reduction in toxicity did not result in a loss of lipid A's adjuvant properties. In addition to this simple modification, it was discovered that removing an ester-linked fatty acid group even further reduced the toxicity of the molecule while retaining immunostimulatory properties [23].

Outer Membrane Vesicles

Outer membrane vesicles (OMVs) are non-replicating components of both virulent and non-virulent gram-negative bacteria. They are comprised of outer membrane lipids, outer membrane proteins, LPS, and soluble periplasmic components, which are compartmentalized into the lumen [24, 25]. Figure 4 illustrates how outer membrane vesicles bleb off the surface of a cell. Because vesicles contain sugars, proteins, and lipids, all of which are foreign to a mammalian host, vesicles themselves have been found to be highly immunogenic [26].

OMVs may serve as an appropriate vaccine delivery system because, as several recent studies have shown, mammalian cells internalize them [27, 28], which will be important for delivering vaccine components throughout the body. Additionally, OMVs of infectious bacteria have been found in the fluids of hosts, suggesting that they are much more permeable than larger bacterial components, delivering their components throughout a host [29]. One study focused on enterotoxigenic *Escherichia coli* (ETEC), which is a cause of traveler's diarrhea, and the delivery of certain toxins into eukaryotic cells via vesicles. It was discovered that hosts show elevated levels of heat-labile enterotoxin (LT), which is involved in the attachment of the vesicles to mammalian cells through an interaction with a host surface receptor, G_{M1} [28, 30]. Another similar study showed that LT is actually enriched in OMVs and suggested that OMVs are a pathogenesis mechanism for ETEC [31]. Additionally in the same study, it was discovered that these vesicles deliver their components into the cell after internalization [28]. Another group similarly showed that vesicles from *Pseudomonas aeruginosa,* a pathogen that is common in cystic fibrosis and other immuno-compromised patients, are internalized into lung tissue to deliver proteases [27]. A curious phenomenon that occurs with vesicles is the enrichment of certain proteins such as virulence factors, toxins, adhesins, hemolysins, and proteases [31, 32]. It is hypothesized that once OMVs are internalized they are eventually trafficked to the endoplasmic reticulum where the proteins are then released into the cytosol as a method for delivering their toxins [29]. This evidence suggests that vesicles may be important in the infection process [24, 25, 33] and that they could be carriers and delivery systems for vaccine components.

It has been demonstrated that OMVs may be engineered to include recombinant proteins in the lumen as well as in the membrane of the vesicles [24, 32]. Kesty *et al* showed that Ail, an adhesin from *Yersinia enterocolitica,* could be

expressed and incorporated into *E. coli* OMVs as shown in Figure 3 [24]. Kim *et al* showed that the outer membrane hemolysin, ClyA, could be fused with GFP and display fully functional GFP on the surface of OMVs [32]. ClyA was chosen based on research that showed that ClyA is naturally enriched in OMVs [34]. The ability to engineer OMVs is critical for the advancement of glycoconjugate vesicle vaccines.

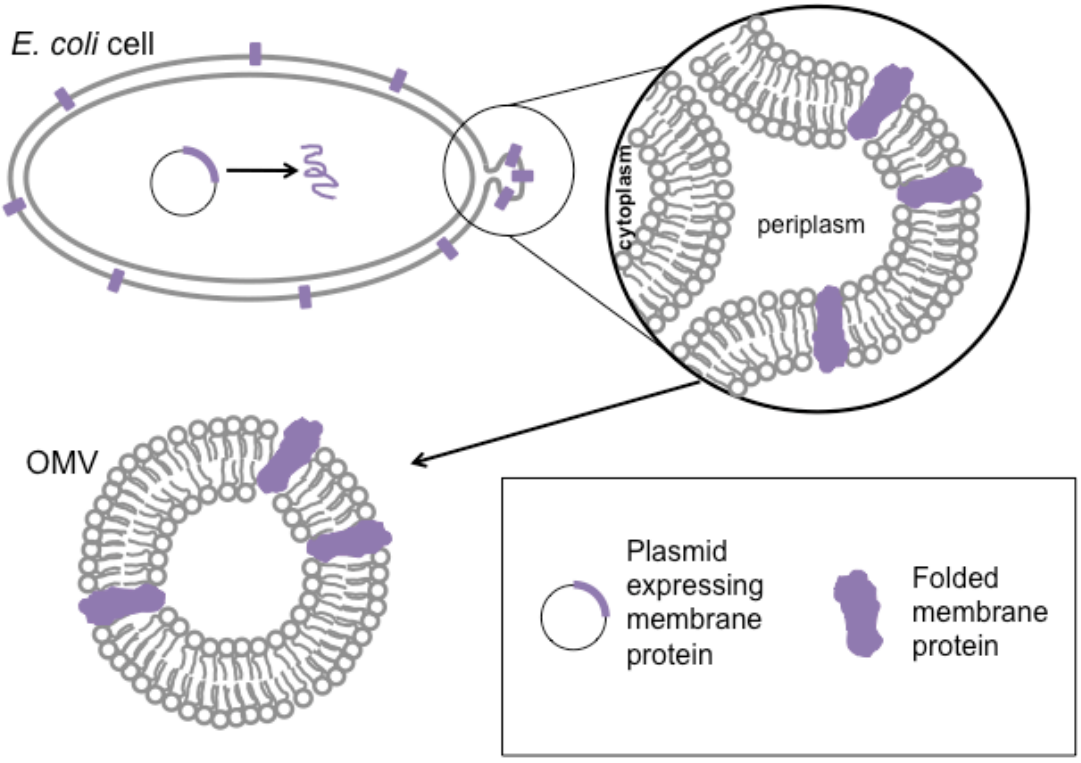


Figure 3: Outer Membrane Vesicle production. Vesicles are formed when the outer membrane blebs and pinches off to create a new vesicle. We have shown that we can express recombinant membrane proteins to localize into these vesicle structures.

As demonstrated recently by Chen *et al* [35], OMVs containing recombinant green fluorescent protein (GFP) fused to ClyA can elicit a potent immune response in mice; whereas exposure to GFP alone without the OMV carrier results in a weak response. ClyA-GFP fusion proteins alone also elicited an immune response, but required more protein than did the ClyA-GFP proteins that were in OMVs. The importance of this study showed that OMVs can able to be a carrier and facilitate protein purification and still retain the ability of ClyA-GFP to elicit an immune response against a GFP, which is normally undetected by the immune system.

Recently, natural OMVs purified from a pathogen itself, *Neisseria meningitidis,* have been extensively studied and accepted as an effective vaccination option for meningitis serotype B [36-39]. These vaccine OMVs are purified through detergent extraction, which eliminates a majority of the LPS which in turn causes the vesicles to lose their structure [40] and to aggregate making them hard to quantify for dosage [41]. While this method reduces the LPS, it also reduces the amount of other immunogenic membrane components and the integrity of the OMVs, thus a small amount of the endotoxin must still remain in the OMVs [42].

This vaccine has been successful in preventing outbreaks of meningitis in Norway, Cuba, and New Zeland [36, 37, 39, 43], but there are a variety of limitations. First of all, the bactericidal serum response is against PorA, which is a variable antigen; the expression of only one type of PorA results in protection against only a specific type of PorA antigen. Mixtures of OMVs from *N. meningitidis* expressing a variety of PorA proteins as well as recombinant DNA techniques so that one strain expresses more than one PorA have been used to overcome this [44]. Some genetic engineering has also been done to optimize the current OMV vaccine technology. *N. meningitidis* has been engineered to increase vesicle production as well as to address the challenges that are associated with the loss of LPS in the detergent extraction. A

mutation to the *LpxL1* gene has been shown to reduce LPS toxicity while still retaining adjuvant properties and eliminating the need to use detergent extraction. This opens up the opportunity to explore other purification methods, such as our method of ultracentrifugation [38, 45]. Finally, the optimization and engineering that has been performed up to date is very specific for this one pathogen.

Oligosacchryltransferase, PglB, and its Function

Previously thought to only occur in eukaryotes, a functional *N*-linked glycosylation locus has been discovered in *C. jejuni* [46, 47]. Szymanski *et al* were able to characterize this *pgl* (*p*rotein *gl*ycosylation) locus and identify homology of these genes to other known eukaryotic genes involved in protein glycosylation [46]. OSTs catalyze the attachment of a lipid-linked sugar to an asparagine residue involved in a consensus amino acid sequence for glycosylation, or glycosylation site, Asn-Xaa-Ser/Thr, on a nascent protein; Xaa stands for any amino acid except proline [48, 49]. These genes have been shown to be functional in *E. coli* and are able to glycosylate proteins that we have engineered to include glycosylation tags.

Further, it has also been proven that PglB is able to transfer *O*-antigen sugars onto proteins containing the consensus sequence [50]. Thus we can utilize this OST to attach our antigens to an adjuvant we have engineered for glycosylation. This is of particular importance to our work because our goal is to attach antigens to outer membrane-bound adjuvants *in vivo* such that only OMV purification would be necessary to purify the vaccine components.

One hurdle of engineering sugar conjugation in *E. coli* is the competing, native reaction of *O*-antigen transfer by WaaL, which ligates *O*-antigens the lipid A-core sugar structure. Feldman *et al* have discovered that in WaaL knockouts, where the competing reaction no longer occurs, PglB is able to transfer the *O*-antigens to protein

[50]. This technology is currently being used by a company, GlycoVaxyn, to produce conjugate vaccines *in vivo.*

This Work

OMV vaccines present a solution to a major drawback of current conjugate vaccine technology: their inaccessibility to developing countries both in terms of cost and implementation. The high cost of these vaccines makes them hard to obtain without the aid of programs such as UNICEF [12]. Recently, the World Health Organization supported an anti-diarrheal vaccine, however health officials cited cost as a hindrance for universal vaccination for children. Conjugate vaccines are also very unstable, which may be problematic in areas where proper refrigeration may not be available. Improper temperature storage as well as multiple freeze-thaw cycles degrades the protein and reduces the vaccine's efficacy [12].

Our work is to expand upon the current limited OMV technologies by using recombinant DNA techniques to create an OMV vaccine that are specific to a pathogen of our choosing. We can engineer bacteria to produce OMVs that already carry highly immunogenic membrane proteins and lipid structures, to subsequently conjugate an antigen to an adjuvant *in vivo*. We have studied two potential carriers that both have adjuvant properties. One carrier is *E. coli* lipid A, the second is outer membrane proteins as described in Figure 4.

We have engineered the *E. coli* membrane protein, ClyA, to contain four glycosylation sites at the C-terminus of the protein. When this engineered protein is expressed in *E. coli* with pglB and the genes that encode the polysaccharide, a glycoprotein is detected in OMV fractions. The antigen-adjuvant complex was created *in vivo* which greatly simplified the purification process to a single ultracentrifugation step. Additionally we did not have to handle any pathogens or use

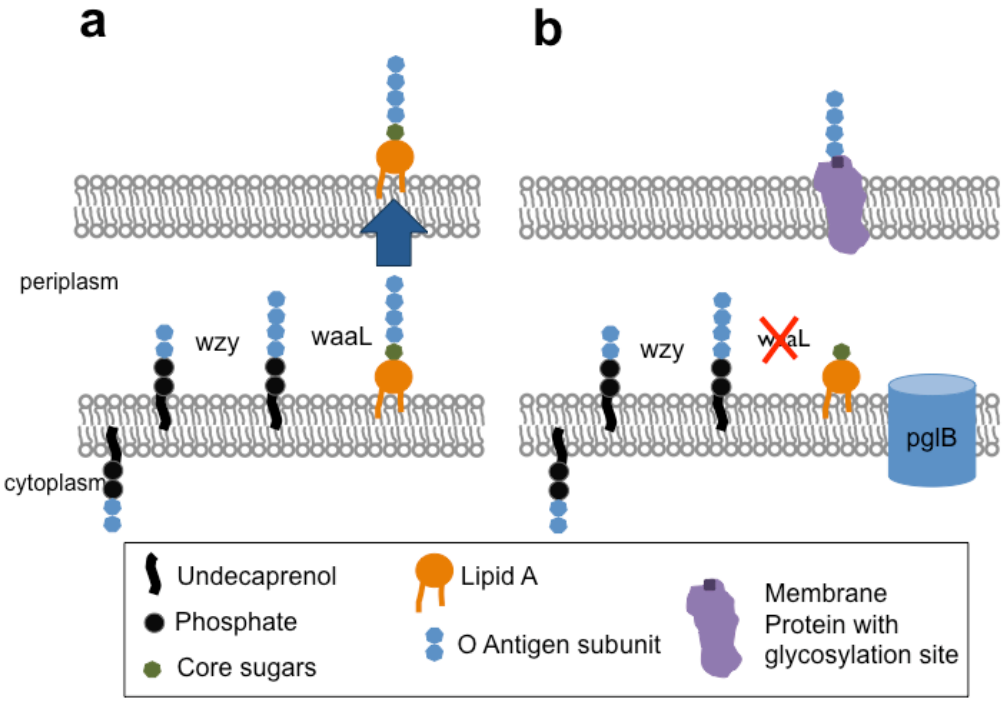


Figure 4: Two ways of decorating OMVs with O-antigen polysaccharides. (a) demonstrates how *O*-antigens (and *N*-glycans) are attached to the lipid A and the core sugars and then localize to the outer membrane by an unknown mechanism. (b) depicts how protein glycosylation occurs in theory with the presence of pglB, a membrane protein engineered to include a glycosylation site, and if the competing, native reaction performed by WaaL is eliminated. Both methods describe ways to localize lipid A and protein conjugated polysaccharides to the outer membrane where they can bleb off with vesicles.

any toxic chemicals that are currently required for cross-linking antigens and protein adjuvants. Choosing to glycosylate a membrane protein that localizes in OMVs builds upon the platform that GlycoVaxyn currently employs by facilitating purification. OMV purification requires fewer steps than protein purification and can be purified from the supernatant.

Additionally, given the approval of the lipid A derivative, MPL as an adjuvant, we studied the conjugation of *O*-antigens to lipid A. We used lipid A in our preliminary work because it is already produced in *E. coli*; we are currently taking the

next steps towards genetically modifying *E. coli* to have a structure more like MPL. Lab strains of *E. coli* already contain the ligation machinery necessary to conjugate *O*-antigen to lipid A and thus *E. coli* only require the gene cluster of a heterologous *O*-antigen. We have shown that we can decorate OMVs with many different O-antigens from many species.

CHAPTER 2: MATERIALS AND METHODS

Bacterial Strains and Plasmids

Table 1: Plasmids used in this work

	Description	Source
pClyA-Glyc-His6	ClyA-4 glycosylation sites-6 histidine tag, Amp^R	This work
pPgl-wt	pgl operon with pglB wt, Cm^R	[51]
pPgl-mut	pgl operon with pglB mut, Cm^R	[51]
pLPS2	*Pseudomonas aeruginosa* O11 antigen gene cluster	[52]
pJHCV32	*E. coli* O7 antigen gene cluster	[53]
pSS37	*Shigella dysenteriae* O-antigen gene cluster	[54]
pPM2212	*Shigella flexneri* O-antigen gene cluster	[55]
pAY100	*Yersinia enterocolitica* O3 antigen gene cluster	[56]
pFTO3	*Francisella tularensis* O3 antigen gene cluster	Rebecca Thomas

Plasmid pClyA-Glyc-6His was constructed by ligating the PCR-amplified clyA gene into pTRC99-glyc-6His between SacI and XhoI sites. pTRC99-glyc-6His contains four repeating consensus sites necessary for glycosylation between XhoI and SalI sites as well as 6 histidines between SalI and HindIII sites. The entire construct was then cut and ligated into pBAD18 using SacI and HindIII sites. Plasmid pClyA-Glyc-6His-pgl or pClyA-Glyc-6His-pglmut were constructed by PCR amplifying the clyA glyc- and histidine-tagged section of pClyA-Glyc-6His and ligating into

pBAD18 using SacI and XmaI sites. pglB and pglB mut were cut and ligated using XmaI and SbfI sites.

Table 2: Bacterial strains used in this work

	Description	Source
JC8031	Hypervesiculating *E. coli*	[57]
CE8032	waaL::Kan, derived from JC8031 a hypervesiculating *E. coli* strain	This work

Strain CE8032 was made via P1 transduction by infecting JC8031 cells with P1 phage from the donor Keio waal::Kan.

P1 Transduction

P1 transduction is a method that takes advantage of a bacteriophage that packages genomic DNA into its capsule. To achieve *waaL* knockouts, P1 phage transducing particles from Keio collection was used. Overnights of the appropriate Keio strain (Keio waaL::Kan), were subcultured 1:100 into 2 mL LB, 5 mM $CaCl_2$, 10 mM $MgSO_4$, 0.2% glucose for approximately 90 minutes at 37˚C, 225 rpm. 50 uL of P1 phage was added to the culture and incubate for 1-4 hours at 37˚C, 225 rpm. After the cultures showed clearing, 10-20 uL of chloroform was added to kill any remaining cells, vortexed and allowed to incubate at room temperature for 10 minutes. After transferring to a clean Eppendorf tube, the culture was spun for 10 minutes at 13,000 rpm. The supernatant containg the phage was collected. 600 uL of overnight of the recipient strain, JC8031, was spun down at 3000 rpm for 2-3 minutes. The cells were resuspended in 100 uL LB, 5 mM $CaCl_2$, 10 mM $MgSO_4$, and 0.2% glycose. After adding100 uL of lysate, the mixture was incubated at 37˚C with gentile agitation. After 30 minutes, 100 uL of 1M sodium citrate and 1 mL of LB were added and allowed to shake for another hour. The cells were then spun down at 3000 rpm for 5

minutes and resuspended in 100 uL LB, 100 mM sodium citrate and plated on LB agar plates with Kanamyacin and 100 mM sodium citrate.

Soybean Agglutinin Labeling

Soybean agglutinin (SBA) conjugated to Alexa Fluor was used to test whether or not the knockouts were achieved by assessing the ability of SBA to attach to available *N*-glycan. 100 uL of overnight cultures containing pPgl were rinsed with PBS and resuspended in 10 μg/mL SBA dissolved in PBS. After incubating for an hour at room temperature, cells were rinsed again with PBS, resuspended in PBS, and analyzed using FACS.

Preparation of OMVs

OMVs were purified using the established procedure previously described by Kolling et al [58]. CE8032 was sequentially transformed first with pClyA-Glyc-His6 and selected on LB-ampicillin plates. These transformants were then transformed with pPgl-wt and pPgl-mut and selected on LB-ampicillin-chloramphenicol plates. Flasks with 50 mL LB media with ampicillin, chloramphenicol, and 0.2% glucose were inoculated with an overnight culture and allowed to grow at 30˚C until an OD_{600} of ~0.4 was achieved. To eliminate the glucose media, cultures were centrifuged for 15 minutes at 3200 rpm, 4˚C. Cell pellets were resuspended in 150 mL LB media with ampicillin, chloramphenicol, and 0.2% arabinose and grown at 30˚C overnight. After 10 hours of induction, cells were centrifuged at 7500 rpm, 20 minutes, 4˚C. Pellets were frozen for His purification. The supernatants were then filtered through a 0.45-μm filter to eliminate any remaining cells. OMV fractions were isolated by ultracentrifugation at 28,000 rpm for 3 hours at 4˚C (Beckman-Coulter; Ti SW28 rotor). Pellets were resuspended in PBS and stored at -20˚C.

Protein Purification

Proteins were purified according to the Qiagen Ni-NTA Spin Kit instructions under native conditions. Frozen cell pellets were resuspended in 1 mL lysis buffer supplemented with 1% (v/v) TritonX-100 and 1mg/mL lysozyme and incubated on ice for 30 minutes. Cells were sonicated 4 times for 30 seconds with a 1-minute rest. The sonicated cells were spun down at 10,000g, 4˚C, 20 minutes. Supernatants were run over the columns, washed, and eluted with 250 mM imidazol.

Western Blotting

Both OMV and purified protein samples were analyzed using SDS/PAGE gels. Samples were prepared in the sample loading buffer with β-mercaptoethanol and boiled for 15 minutes at 100˚C. Samples were then run on 12% polyacrylamide gels (BioRad, Mini-PROTEAN® TGX) in duplicate. For western blotting, proteins were transferred onto nitrocellulose membranes and probed the appropriate primary antibody specific for the polysaccharide or anti-6xHis conjugated to HRP (1:5000). Membranes probed with anti-Hr6P were probed with anti-rabbit (1:2500) secondary antibody.

CHAPTER 3: RESULTS

Host Cell Engineering

A *waaL* knockout in the vesiculating *E. coli* strain JC8031 was necessary for two reasons. One, as described by Feldman *et al*, WaaL ligates bactoprenol-linked polysaccharides to lipid A [50]. Because bactoprenol-linked polysaccharides are the substrate that pglB uses, it is necessary to eliminate the competing reaction performed by *waaL* to increase the efficiency of the protein glycosylation performed by pglB. Secondly, we wanted to analyze whether or not the *O*-antigen ligase is in fact *waaL* and whether it has the ability to transfer a variety of polysaccharide structures.

To create the knockout, P1 transduction was utilized by making transducing phage with Keio waaL::Kan. The transducing phage was used to infect JC8031 and the colonies that conferred Kanamyacin resistance were chosen. In order to test the knockout, the Keio parent, Keio waaL::Kan, JC8031 and the putative JC8031 waaL::Kan were transformed with the pgl operon which contains the genes that express the *N*-glycan. These cells were grown overnight and labeled with SBA and analyzed using FACS. Cells that have a functional WaaL will be able to transfer the *N*-glycan to the lipid A which subsequently localizes to the surface so that SBA can bind and label the cells for FACS analysis. If the *waaL* knockouts were successful, the *N*-glycan cannot be transferred to lipid A and there would be no fluorescent signal. The results shown in Figure 5 show a loss in fluorescent signal in the JC8032 waaL::Kan, renamed CE8032.

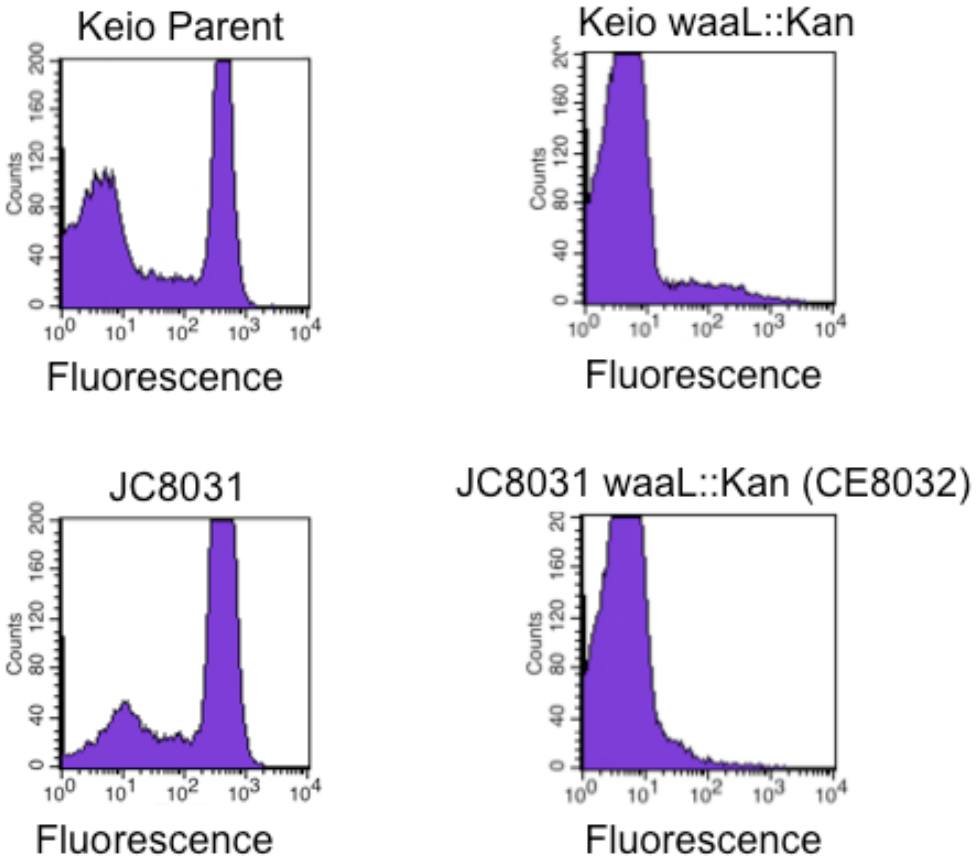


Figure 5: Knocking out *waaL* in JC8031. FACS results measuring for fluorescence in SBA labeled cells. All four panels represent different cell lines expressing the pgl operon. The Keio parent and JC8031 show fluorescent signal as expected when N glycan is localized to the surface due to waaL ligation. Keio *waaL*::Kan and JC8031 *waaL*::Kan show a loss in signal when *waaL* is knocked out and N glycan is not attached to lipid A before export to the outer membrane.

O-antigen Expression and Lipid A Conjugation

As described before, *O*-antigen sugar structures are widely varied across bacteria. We received a variety of *O*-antigen gene clusters from various species. It was important to test not only the expression of these antigens but if they could localize to OMVs. All of the vectors were transformed into JC8031, grown, and OMV fractions were isolated and run on SDS-PAGE polyacrylamide gels. Each antigen we tested had specific antiserum for western blotting. As shown in Figure 6, we tested the *N*-glycan from *C. jejuni*, and the *O*-antigens from *E. coli, Pseudomonas aeruginosa, Yersinia enterocolitica, Shigella dysenteriae, Shigella flexneri,* and *Francisella tularensis.* The first lanes of each blot are JC8031 expressing the empty vector that the *O*-antigen was cloned into or just OMVs isolated from JC8031, depending on whether or not we had the empty vectors. The second lane in each blot is JC8031 expressing the *O*-antigen genes. Each example shows banding, which is very common in *O*-antigens as they are often polymers of repeating sugar structures.

We theorized that WaaL was responsible for attaching the polysaccharides to the lipid A. In order to analyze whether or not WaaL has an integral part in attaching *O*-antigens and N glycan to create LPS, the *O*-antigen gene clusters were expressed in the *waaL* knockout strain CE8032. If WaaL is responsible for attaching the *O*-antigen to complete the LPS structure, a knockout would result in a loss of antibody attachment in a blot because presumably the *O*-antigens had no way of localizing to the outer membrane or OMVs. The third lane in all of the blots, except for in Figure 6d (*Y. enterolitica)*, is the *O*-antigen expressed in CE8032. The cosmid for the *Y. enterocolitica* O-antigens would not express in CE8032. The loss of antibody attachment in these lanes provides evidence that WaaL is in fact responsible for the attachment of *O*-antigen to complete the LPS structure. This further implicates that

WaaL is promiscuous enough that it was able to recognize all of the O-antigen structures as a substrate and attach them to the core sugars.

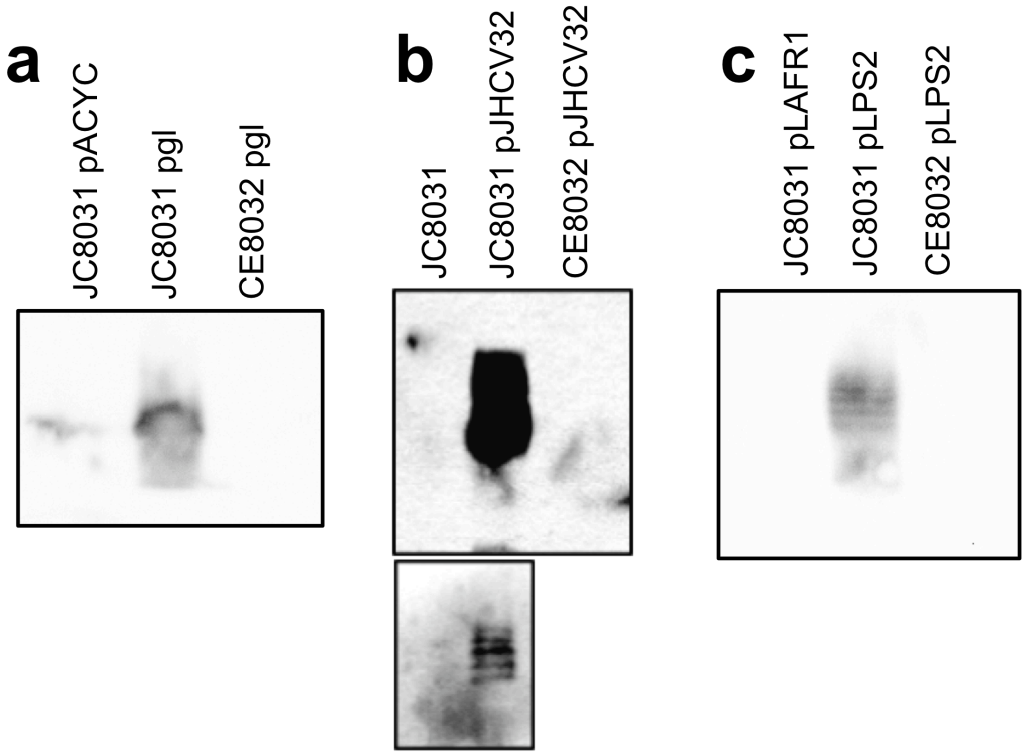
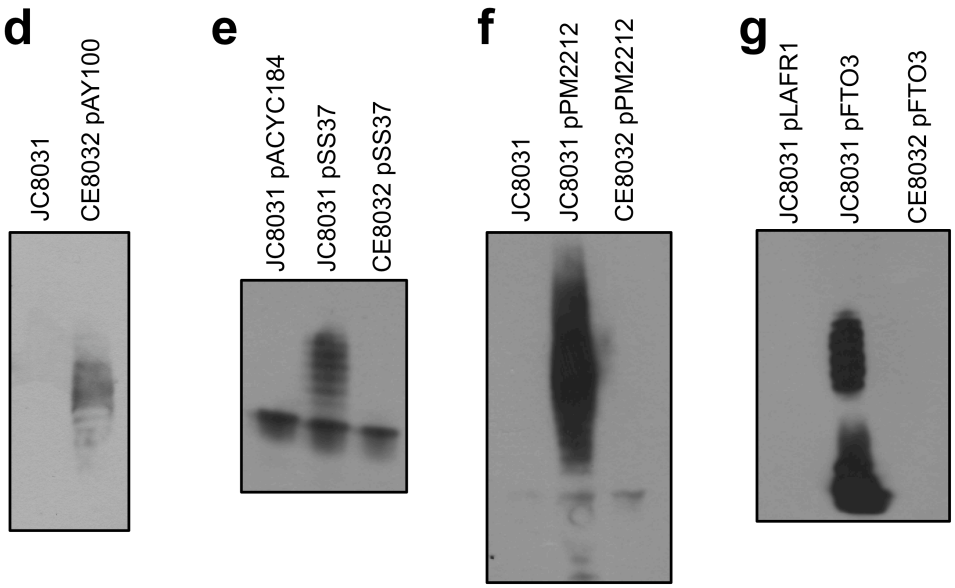


Figure 6: Lipid A conjugated *N*-glycan and *O*-antigens. All lanes represent OMV fractions and were western blotted with commercial antiserum specific for the polysaccharide being studied. (a) *C. jejuni* N glycan. (b) *E. coli* O7. (c) *P. aeruginosa* O11. (d) *Y. enterocolitica* O3. (e) *S. dysenteriae* O-antigen. (f) *S. flexneri* O-antigen. (g) *F. tularensis* O-antigen. In (a-g) the first lane is JC8031 cells with the empty vector that the polysaccharide genes were cloned into or JC8031 without any plasmid as indicated. The second lane is JC8031 expressing N glycan or O-antigens. The third lane in (a, b, c, e, f, g) is the *waaL* knockout expressing N glycan or O-antigens. This lane is missing in (d) because the plasmid would not express.

Figure 6 (Continued)



Protein Conjugation

Proteins are known adjuvants and are a good target for *O*-antigen conjugation to produce a conjugate vaccine *in vivo.* Thus, ClyA, a membrane protein that is a known adjuvant, was engineered to carry four glycosylation consensus sites, also known as a glyc site. The ClyA was also engineered to include a 6x histidine residue for purification as well as western blot detection. We expressed this engineered ClyA with the pgl operon, which includes the genes for making the *N*-glycan and pglB for

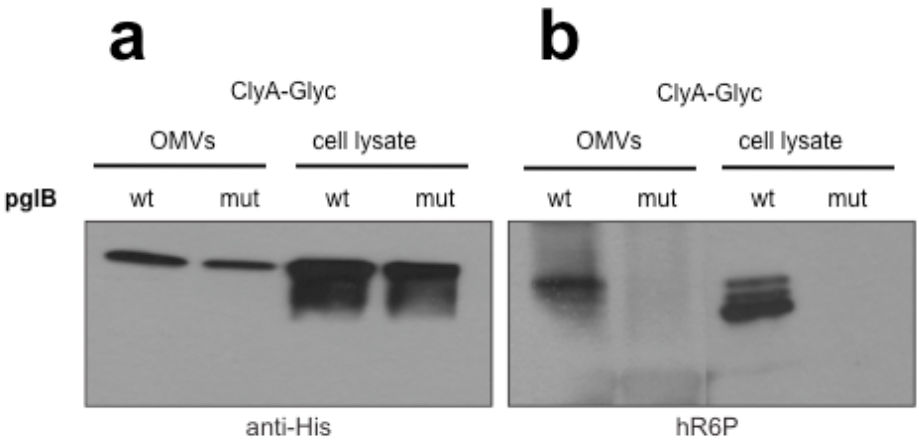


Figure 7: Protein glycosylation in OMVs. The first two lanes in (a) and (b) are OMV fractions where the pgl operon was expressed with either pglB wt or pglB mut as indicated with pClyA-Glyc-6His. The anti-His blot demonstrates that both proteins expressed well. The hR6P blot demonstrates that the N-glycan is only present when there is a functional pglB. The last two lanes in (a) and (b) are purified protein from whole cell lysates verifying that ClyA is in fact a glycoprotein when pglB is functional.

transferring the *N*-glycan to a protein, in CE8032. We also expressed the engineered ClyA with the pgl operon mutated, which has functional *N*-glycan synthesis genes, but a non-functional pglB. CE8032 was chosen because it was a hypervesiculating *E. coli*

that also had *waaL* knocked out. In an attempt to maximize the glycosylation that occur, we wanted to use a *waaL* knockout to eliminate the competing, native reaction.

The experiments that contained a pglB wt should show glycosylation on ClyA whereas the pglB mut should show none. Glycosylation was detected using western blotting with antiserum specific for the N-glycan, anti-Hr6P. Protein expression was detected using anti-6His. The blot in Figure 7 shows both the OMV fractions as well as the purified protein from whole cell lysates. The first two lanes in (a) and (b) are OMV fractions, with the first expressing pglB wt and the second expressing pglB mut. Based on these results, it is clear that the protein is expressing with bands appearing in both lanes in the anti-His blot. The anti-Hr6P blot shows a distinctive banding pattern in the pglB wt and none in the pglB mut case showing that the pglB wt was functional in transferring N-glycan to the glyc tagged ClyA. Further to be sure that the protein itself was being glycosylated the third and fourth lane are the purified protein from whole cell lysate using Ni-NTA columns. Again, these results show that the protein was expressed well in both cases and showed that N-glycan was attached to these proteins in the pglB wt and not in the pglB mut experiment.

CHAPTER 4: DISCUSSION

The results from this work have shown that we can decorate OMVs with polysaccharides using two different conjugation methods that each have the potential to be vaccine candidates. While OMVs are currently being used as vaccines and some genetic engineering has been done to optimize the process, those vaccines are only for meningitis serotype B and optimizations are very specific for OMVs produced by *N. meningitidis* [44, 45]. This work has been focused around developing a strategy for decorating OMVs with polysaccharides that can be universal and robust.

We have shown that we can glycosylate and recover membrane proteins *in vivo* by isolating OMV fractions. The *in vivo* glycosylation of antigens to protein adjuvants to make conjugate vaccines is currently being developed by the company GlycoVaxyn. While their technology has simplified the conjugate vaccine production process to that shown in Figure 1b, the protein purification process is still costly and the product still needs to be properly stored or the proteins could degrade decreasing its efficacy [12]. Our work hopes to expand upon this technology by glycosylating membrane proteins. By using a membrane protein that is embedded and protected in the outer membrane, we can potentially reduce the purification process to that of ultracentrifugation or ultrafiltration. Furthermore, the OMV is a stable carrier for the glycoproteins as well.

We have also shown that we can express a variety of *O*-antigens from various species in *E. coli* and that they do localize in OMVs. Our system seems to be universal enough where many types of *O*-antigens are able to be expressed in *E. coli* and thus could imply that this could be a promising method. Lipid A is known to be an adjuvant, but in certain quantities, is extremely toxic. While some studies have

suggested that membrane-bound lipid A is less toxic than free lipid A, the current OMV vaccine technology for meningitis was developed using detergent extraction which eliminates much of the lipid A. Because of the problems associated with detergent extracted OMVs, there is research being performed to find alternative purification methods while still ameliorating the toxic effects of lipid A. Current information suggests that modified lipid A structures, such as MPL or *LpxL1* mutants or knockouts, are an equally effective adjuvant at eliciting an antibody immune response while also being less toxic and can be purified using ultracentrifugation [20, 45].

We have proven that *E. coli* already contains the necessary ligase, WaaL to attach the *O*-antigens examples we tested onto lipid A. We hope that because of the variety of antigens WaaL conjugated to lipid A, that this could be a universal method for creating antigens conjugated to the adjuvant lipid A. In every *O*-antigen expressed, the host was the same non-pathogenic JC8031 *E. coli* strain. In combination with the data we have generated and the information on modified lipid A structures, we could engineer a non-pathogenic *E. coli* to naturally produce modified lipid A structures that are already being used as adjuvants. These *E. coli* should have the functionality to produce non-toxic, adjuvant lipid A structures but also retain its ability to conjugate *O*-antigens to these structures *in vivo* to create safe, effective OMV vaccines.

We believe that our two conjugate vaccine methods gave the potential to be effective bacterial vaccines. Both options attempt to optimize and facilitate the production of current vaccine technologies. By combining the current knowledge on OMV vaccines, modified lipid A structures as adjuvants, and conjugate vaccines, we believe we have presented a way to combine and optimize the best of each of these vaccine technologies.

CHAPTER 5: FUTURE WORK

Our future work involves further developing these methods to create a new class of bacteria vaccine. One aim is to engineer a host strain that produces OMVs that are non-toxic to humans yet still has adjuvant properties, carry *O*-antigens, and can elicit a proper immune response. In order to further the results discussed here into a technology, we must optimize our OMVs to elicit the ideal immune response, expand the scope of our *O*-antigens, and to characterize OMVs.

We would like to develop methods to clone gene clusters of *O*-antigens. While we have received a diverse array of isolated *O*-antigens in vectors from collaborators, we are limited by the small fraction of *O*-antigen genes that have actually been isolated and cloned. We would like to develop methods to be able to clone these gene clusters from genomic DNA of pathogens and be able to remove these expand the scope of expression of *O*-antigens. *Vibrio cholerae* is one example of an *O*-antigen that would be of interest to express for vaccine development, but could not express from what we were given by collaborators. This is an important method to develop not only because we could address any new pathogens that may arise, but because it would further prove our work as a method for expressing virtually any *O*-antigen in *E. coli.*

Other optimization that should be performed is in finding an appropriate adjuvant protein carrier for the glycosylation of protein adjuvants. We used ClyA as our model system because it is a known outer membrane protein and an adjuvant, but it is also a hemolytic protein [59], which in certain dosages may prove to be harmful to a patient. ClyA mutants are being screened for their localization abilities as well as if they retain their adjuvant properties. Additionally, the location of the glycosylation site should be studied. At this time, it is unknown if the glycosylated segment of the

protein is outside the vesicle or in the lumen. Further characterization should be performed to analyze whether or not the adjuvant-antigen complex would be effective given the current glycosylation site. We would want to optimize not only the protein but the location of the antigens.

One way we propose to reduce the toxicity of our OMVs is to mutate or knockout specific genes involved in lipid A synthesis that would eliminate any toxic effects. A screen should be done to identify candidates for genetic knockouts or modifications that could mitigate the toxicity of lipid A while retaining its adjuvant properties. For example, *LpxL1* knockouts in *N. meningitis*, have already been identified as a potential target [45]. Further, understanding lipid A synthesis and the genes involved could be another way to alter the *E. coli* lipid A so that it is non-toxic. In addition to screening *E. coli* mutants for less toxic lipid A, another possibility would be genetically modify *E. coli* to knockout certain lipid A construction steps in order to create an MPL-like structure *in vivo.* The difference between the *E. coli* lipid A structure and the MPL structure is shown in Figure 8. Various bacteria, such as *Helicobacter pylori* and *F. tularensis*, modify their lipid A structures in order to evade the human immune system [60, 61], thus there are enzymes that could perform these modifications in *E. coli.* A major difference between *E. coli* lipid A and non-toxic MPL is a single phosphate group. Both *H. pylori* and *F. tularensis* modify their lipid A with enzymes that cleave the phosphate group that would make *E. coli* lipid A more chemically similar to MPL [60, 62]. If we can delete the genes for the enzyme that is responsible for adding that phosphate group it would eliminate any post-purification detoxification that is currently used to create MPL.

In addition to trying to engineer *E. coli* to produce non-toxic lipid A, we are looking into other forms of *E. coli* as our host strain. Recently we discovered that we can engineer an *E. coli* that was isolated from human intestines, Nissle1917, to

vesiculate. Because these bacteria live naturally in human intestines, we hypothesized that these *E. coli* are somehow not as toxic to our systems as lab strains of *E. coli* would be, but this has not been tested. Nissle1917 has the same *E. coli* lipid A structure so it still could be possible that these strains would require more engineering to alter their lipid A structures as previously discussed.

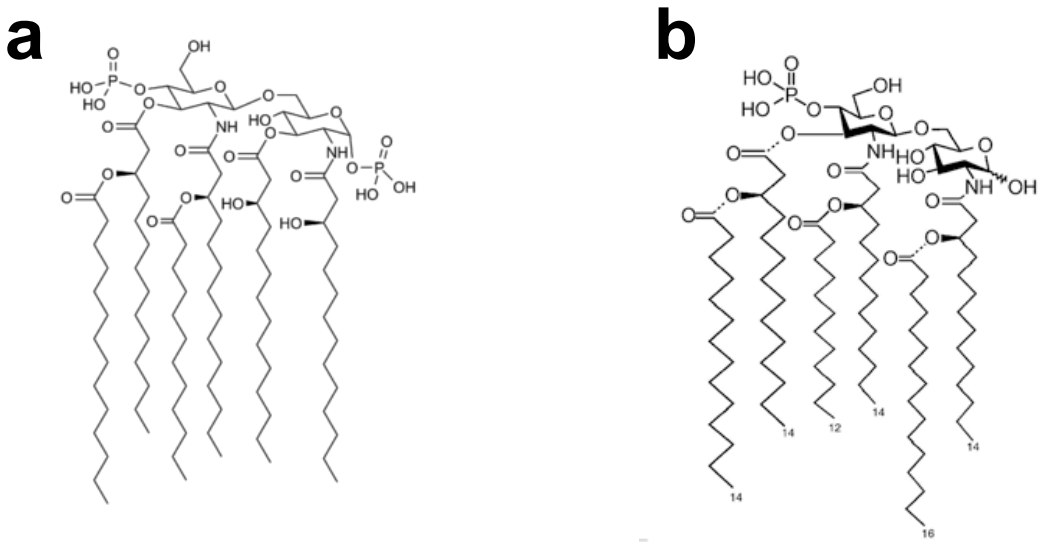


Figure 8: *E. coli* lipid A and MPL. (a) depicts the *E. coli* lipid A structure [1] (b) depicts the MPL structure [4]

Experiments are currently being performed to analyze the difference in immune response in mice of OMVs and LPS with the same *O*-antigen attached. We hypothesized that because the OMVs have many other components that could serve as adjuvants that the OMVs could potentially reduce the total amount of lipid A that must be administered to elicit an immune response. If these experiments prove that OMVs can elicit more antibodies than the same effective does of lipid A in LPS, this could be a first step towards proving lipid A, or modified lipid A, conjugated vaccines could be effective.

All of the future work proposed aims to create OMVs that can be produced *in vivo* to reduce processing steps and to eliminate the need to handle pathogens. Our

aim is to ultimately develop an OMV vaccine technology that will be widely universal and that exploits the benefits of vesicles to create a cheap, effective, accessible vaccine.

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