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The Effect of O Antigen Loss on the Protein Composition and Inflammatory Response Elicited by Klebsiella pneumoniae

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The Effect of O Antigen Loss on the Protein Composition and Inflammatory Response Elicited by *Klebsiella pneumoniae*

by

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Abstract: *Klebsiella pneumoniae* is a Gram-negative pathogen associated with numerous infections. Like all Gram-negative bacteria, *K. pneumoniae* naturally release outer membrane vesicles (OMVs) during all stages of cellular growth. OMVs are composed of the outer membrane components such as lipopolysaccharide (LPS) and outer membrane proteins and contain cytosolic and periplasmic proteins in the lumen. *K. pneumoniae* is often found to lack an O antigen. The absence of the O antigen has been reported to alter the protein content of the membrane which may further alter the immune response elicited by *K. pneumoniae*. Therefore the purpose of this study was to analyze the protein content and inflammatory responses of the cellular outer membrane (OM) and the secreted OMVs. A secondary goal of this study was to evaluate any changes to the membrane protein content due to O antigen loss and determine if the loss of the O antigen influences the inflammatory response. This study demonstrated that the cellular OM and OMVs have distinct protein profiles as well as inflammatory responses. OMVs were highly enriched in outer membrane proteins compared to the cellular OM and had a significantly stronger inflammatory response. The protein content of the OM and OMVs were further modified by the absence of the O antigen from the outer membrane. Although there were no significant differences seen in the wild type and O antigen deficient OM inflammatory responses, the loss of the O antigen resulted in OMVs that produced a significantly stronger IL-6 response.

Chapter 1

Introduction

Klebsiella pneumoniae is a nosocomial pathogen associated with numerous infections ranging from mild urinary tract infections to bacteremia, pneumonia, and pyogenic liver abscess (1-3). This pathogen has become resistant to many late generation antibiotics and hospital acquired *K. pneumoniae* infections have a 50% mortality rate if left untreated (2, 4). Although *K. pneumoniae* is a prominent nosocomial, multi-drug resistant pathogen, there is a general lack of knowledge with regards to the pathogenesis of and immune response to *K. pneumoniae*.

K. pneumoniae has several virulence factors including an anti-phagocytic capsular polysaccharide (CPS), lipopolysaccharide (LPS), and adhesins (5). LPS is important for the maintenance and integrity of the outer membrane and contains molecular structures that serve as recognition molecules for the immune system. It is considered the most potent stimulator of the innate immune response to Gram-negative, enteric bacteria such as *Klebsiella pneumoniae* (6). The LPS is a three part structure consisting of the lipid A, core oligosaccharides, and the O antigen (Figure 1.1) (7). The lipid A is integrated into the bacterial outer membrane and has a structure that is conserved among Gram-negative, enteric bacteria (6). The lipid A is the pathogen associated molecular pattern (PAMP) for toll-like receptor 4 (TLR-4) on the surface of phagocytic cells, triggering production of inflammatory cytokines (8, 9).

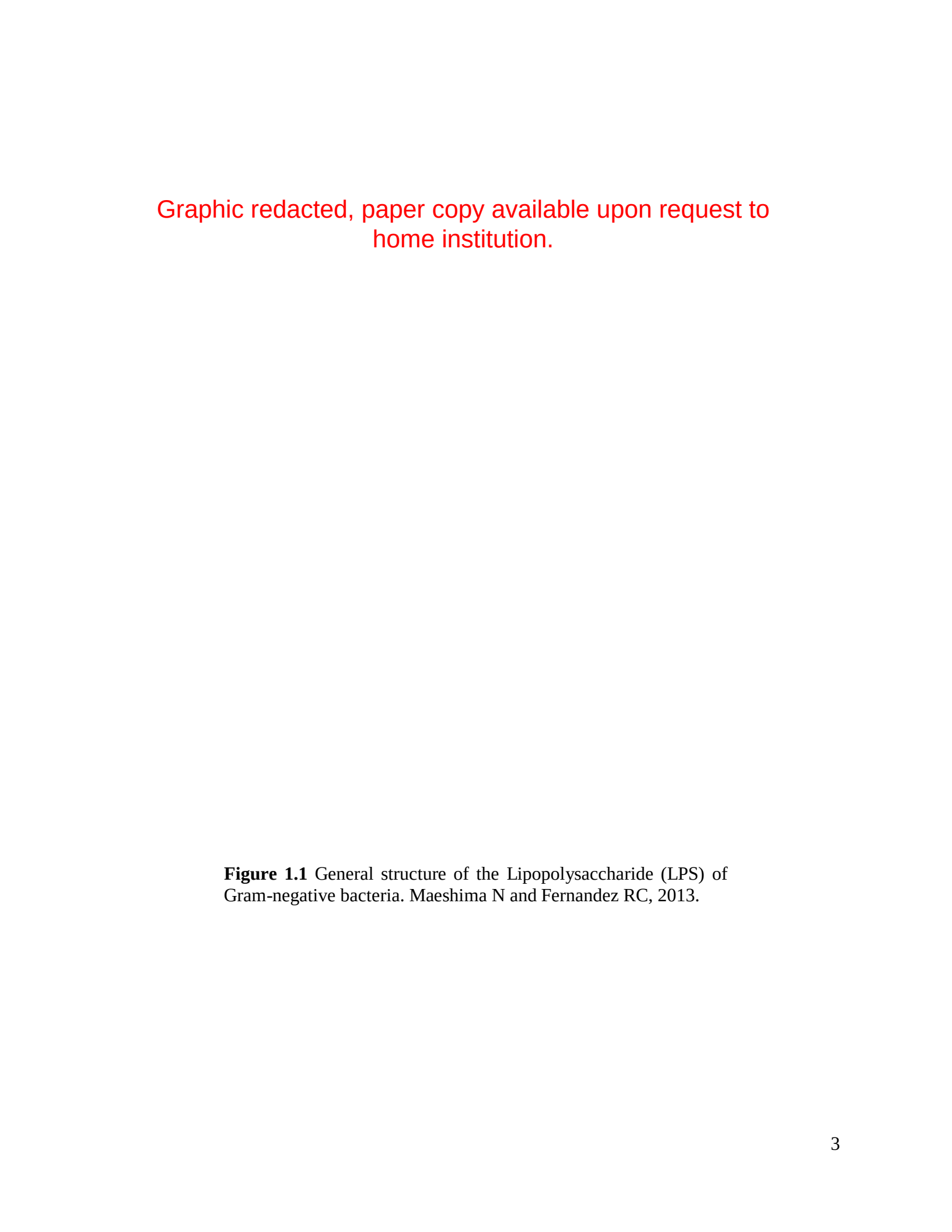

Figure 1.1 General structure of the Lipopolysaccharide (LPS) of Gram-negative bacteria. Maeshima N and Fernandez RC, 2013.

The O antigen consists of several (up to fifty) repeating carbohydrate subunits linked to the core polysaccharide of the LPS molecule (8, 10). Each repeating unit is composed of up to eight hexose sugars that differ based upon sugar, sequence, linkage, and ring forms (8). The O antigen is variable between and within Gram-negative species. Because the O antigen is the outer most portion of the bacterial cell structure, it is recognized and targeted by the host's adaptive immune response. This non-protein antigen stabilizes the bacterial cell wall, helps regulate permeability, and protects the bacterial cell by physically hiding other outer membrane components from antibodies and complement proteins (11).

Although the O antigen has stabilizing properties, the O antigen can be truncated or absent in Gram-negative bacteria (6). For example, *Escherichia coli* and *Salmonella thyphimurium*, can exhibit mutations in the genes responsible for the synthesis and attachment of the O antigen and produce rough LPS mutants lacking their O antigens (12). A serotype analysis of over 600 *K. pneumoniae* clinical isolates obtained from the United States, Denmark, and Spain determined that roughly 14% of the isolates completely lacked the O antigen from their cell surface (11, 13). *K. pneumoniae* is only one of several Gram-negative pathogens in which O antigen loss has been identified. Cystic fibrosis patients have been observed with O antigen-deficient strains of *Pseudomonas aeruginosa (14)*. *Yersinia pestis*, the causative agent of the plague, is an exclusively O antigen-deficient bacterium (15). Other pathogens, such as strains of *Acinetobacter baumannii* and *Neisseria meningitis*, have been isolated lacking LPS entirely (16, 17). Even though *A. baumannii* and *N. meningitis* lack the entirety of the LPS molecule, these pathogens are still capable of stimulating a potent inflammatory response *in vitro*.

It has been established that *K. pneumoniae*, among other Gram-negative bacteria, naturally secrete LPS and other cell derived components through the production of outer

membrane vesicles (OMVs) (18). OMVs are 20-200nm spherical bilayers released from the outer membrane of Gram-negative bacteria during all phases of growth (Figure 1.2) (19). OMVs can serve as decoys that aide in bacterial survival by releasing toxic compound from the cells. OMVs remove over-expressed or mis-folded proteins to relieve bacterial envelope turgor pressure, and OMVs can also release attacking phages and antimicrobial peptides that bind to the membrane (20). OMVs are involved in cell-to-cell communication and have been known to be involved in the transfer of antibiotic resistance enzymes and horizontal gene transfer between bacterial species (21, 22).

OMVs form when part of the outer membrane protrudes and is released from the cell. Therefore the contents of the OMVs are reflective of the outer membrane containing LPS, phospholipids, and outer membrane proteins with cytosolic and periplasmic proteins trapped in the vesicle lumen (Figure 1.2) (18, 23, 24). Although the OMVs are derived from the cellular outer membrane, the protein content of OMVs are distinct from the outer membrane. This has been confirmed by visualization of the protein content of the cellular outer membrane and secreted OMVS indicate that OMVs have protein profiles that are dissimilar to those of the outer membrane (25-27). The enrichment or exclusion of protein cargo into OMVs has been well demonstrated by many pathogenic bacteria that selectively package virulence factors into OMVs (26, 28). For example, Enterotoxigenic *Escherichia coli* (ETEC) exclusively packages heat labile enterotoxin into OMVs (16). In the dental pathogen *Porphorymonas gingivalis*, ninety five percent of the gingipain protease produced by this pathogen becomes packaged into OMVs (28). These studies indicate that a selective sorting mechanism may exist for inclusion of protein cargo into OMVs; however, the current OMV proteomic studies are limited to only evaluating the

protein content of the OMVs and lack a comprehensive proteomic analysis comparing the OMV and outer membrane sub-proteomes.

Figure 1.2 Outer membrane vesicles (OMVs) are released from the outer membrane of Gram-negative bacteria. OMVs contain outer membrane components such as lipopolysaccharide, phospholipid, and outer membrane proteins. Cytosolic and periplasmic proteins are integrated into the lumen of OMVs during vesicle formation. Kuehn and Ketsy, 2005.

The protein content of OMVs is not only distinct from the outer membrane but can be altered by other changes to the outer membrane, such as O antigen loss. It has been established that the absence of the O antigen drastically impacts the protein content of OMVs. *P. gingivalis* strains that lacked the O antigen produced OMVs that carried additional protein and virulence factors that were absent from the source bacterial cell (26). The loss of the O antigen also altered the protein content of OMVs derived from *P. aeruginosa* (25). It has been proposed in several reviews addressing the formation of cargo selection of OMVs that charged O antigen sugar residues may directly interact with certain protein microdomains causing these proteins to be packaged into OMVs (26, 29).

Furthermore, modifications to the outer membrane and OMVs resulting from O antigen loss may also alter their inflammatory response. The LPS is the key immunogen of Gram-negative bacteria and vesiculation is the main LPS delivery mechanism that Gram-negative pathogens employ during an infection. OMVs are enriched in lipopolysaccharide (LPS) and outer membrane proteins that synergistically stimulate the host immune response (18, 30, 31). *K. pneumoniae*- and *P. aeruginosa*-derived OMVs stimulate a potent inflammatory response (30, 31). It has also been demonstrated that rough and smooth LPS elicit different immune responses *in vitro* and *in vivo* (32-35). However, the previously described studies used chemically extracted LPS and it has been shown that vesicle bound LPS and protein cargo produced a significantly stronger inflammatory response when compared to purified LPS (31). Therefore, OMVs should be included when evaluating LPS-related immunological studies.

O antigen loss has been identified in *K. pneumoniae* clinical isolates, therefore, analyzing how O antigen loss influences the ability of OMVs to stimulate the immune response should be considered when studying the pathogenicity of *K. pneumoniae*, since these factors occur

simultaneously during infection. The study performed by Evrard et al. (2010), noted that *K. pneumoniae* strains with LPS mutations were capable of up-regulating cytokine production in dendritic cells (2). However this study only analyzed the effects of O antigen loss on the immune response to *K. pneumoniae* cells and did not include OMVs in their analysis. OMVs are being studied as possible candidates for acellular vaccines and targets for antibody treatments (18, 30, 31). A recent study by Leitner et al. (2013), analyzing possible *Vibrio cholerae* OMV vaccines stated that mice inoculated with OMVs derived from an O antigen-deficient strain of *V. cholerae* were able to produce a more specific, protective immune response than mice inoculated with wild-type vesicles (36). *V. cholerae* OMVs lacking the O antigen were enriched in proteins that were absent from wild-type OMVs. Because these *V. cholerae* OMVs lacked their O antigens and the outer membrane surface antigens were exposed, O antigen-deficient *V. cholera* OMVs produced a stronger immune response than wild-type OMVs (36).

In *K. pneumoniae,* O antigen loss and OMVs have been studied independently of one another. The protein content of the outer membrane is distinct from the OMV content. Studies conducted by Haurat et al. (2011) and Murphy et al. (2014), demonstrated that O antigen loss drastically alters OMV protein content (25, 26). The changes to the membrane and the secreted vesicles produced by the absence of the O antigen could also drastically alter the inflammatory response to *K. pneumoniae*. As seen in the results obtained in the *V. cholerae* OMV vaccine study, both O antigen loss and OMV should be evaluated together when analyzing the immune response to *K. pneumoniae*. Therefore, a comprehensive proteomics-based study was designed to evaluate the differences between the outer membrane and OMV sub-proteomes. This proteomic analysis also analyzed the impact of O antigen loss of the both the membrane and secreted OMVs. The second part of this study focused on how the resulting changes influenced the

inflammatory responses to the cellular outer membrane and OMVs. Specifically, this study addressed whether the inflammatory response differed between the cellular and secreted outer membrane components and whether the loss of the O antigen further altered either of their inflammatory responses. It is anticipated that the protein content of the outer membrane and OMVs will be distinct from one another and therefore potency of the inflammatory response will vary between these different membrane components. It is also anticipated that both the protein content and the inflammatory response of the membrane and OMVs will be further altered by the absence of the O antigen.

Chapter 2

Proteomic Analysis of the Cellular Outer Membrane and Secreted Outer Membrane Vesicles in Wild Type and O Antigen-Deficient *Klebsiella pneumoniae*

Introduction

Gram-negative bacteria naturally secrete lipopolysaccharide (LPS) and other cell derived components through the production of outer membrane vesicles (OMVs) (18, 30). OMVs are 20-200nm diameter spherical bilayers released from the outer membrane of Gram-negative bacteria during all phases of growth (19). Outer membrane vesicles may be used as a method of secretion and delivery for a variety of bacterial virulence factors, including invasion-associated factors, antibiotic resistance enzymes such as β-lacatamase, toxins, and components that modulate host immune responses (23, 28, 29). In fact, some of these proteins, such as the heat labile enterotoxin of enterotoxigenic *Escherichia coli* (ETEC), and the gingipain proteases in *Porphorymonas gingivalis*, are delivered to host cells nearly exclusively by OMVs (26, 28). This selective packaging of cargo into OMVs allows these extracellular structures to function as virulence factors separate from the bacterial cell.

The proteomic profile of OMVs have been evaluated for a number of Gram-negative pathogens including *P. aeruginosa*, *E.coli*, *K. pneumoniae*, *N. meningitidis*, and *A. baumannii*

(37). Most of these studies have focused on the contents of purified vesicles without a side-by-side comparison to the source bacterial outer membrane. These experiments have confirmed that OMVs are enriched in outer membrane and periplasmic proteins, while they also contain cytoplasmic proteins suggesting that a selective mechanism exists to package proteins into OMVs. With the exception of *N. meningitidis* and *N. gonorrhoeae*, most proteomic-based OMV analyses have not directly analyzed or compared the protein content of OMVs to the outer membrane of the originating cells (27, 38, 39). In this study, we aimed to analyze how the OMV sub-proteome compares to that of the outer membrane and to identify the key protein components that are selectively packaged from the outer membrane into the OMV sub-proteome.

For this study we focused on the membrane and vesicles from *Klebsiella pneumoniae*, a nosocomial pathogen associated with numerous infections such as urinary tract infections, bacteremia, pneumonia, pyogenic liver abscess, and ankylosing spondylitis (1-3, 40). Hospital acquired *K. pneumoniae* infections have a 50% mortality rate if left untreated, in large part due to the rapid acquisition of antibiotic resistance, adding to the challenge of treatment of this infection (2, 4). Given this high level of clinical significance, there is scant knowledge of virulence mechanisms for this organism other than the capsule. There has been only one published study to date analyzing the pathogenicity and protein composition of *K. pneumoniae* OMVs (30); which used a limited dataset of 159 proteins to focus primarily on the ability of *K. pneumoniae* OMVs to elicit an inflammatory response (30). Therefore, basic knowledge of the protein components most enriched in OMVs from this clinically significant species remains uncharacterized.

Beyond the protein components, both the bacterial outer membrane and outer membrane vesicles are defined by the presence of lipopolysaccharide (LPS). LPS is a three part macromolecule consisting of the lipid A, core oligosaccharides, and the O antigen (41).

Modification to the structure of this molecule is a well-documented mechanism of enhancing bacterial virulence and survival. For example, lipid A modifications or complete loss of the LPS have been identified in colistin-resistant species such as *Acinetobacter baumannii* (16). The O antigen consists of up to fifty repeating carbohydrate subunits linked to the core polysaccharide of the LPS molecule (6). This carbohydrate structure functions to stabilize the bacterial cell wall, regulate membrane permeability, and protect the bacterial cell by physically hiding other outer membrane components from antibodies and complement proteins (11). Because the O antigen is the outer most portion of the bacterial cell structure, it is recognized and targeted by the host immune system.

Loss of the entire O antigen structure can result in reduced immune recognition of these bacterial pathogens. O antigen-deficient clinical isolates of *Yersinia pestis* and *P. aeruginosa* isolates from cystic fibrosis patients have been observed (14, 15). A study analyzing the prevalence of O antigen serotypes in 638 *K. pneumoniae* clinical isolates reported that 8.3 % were O antigen-deficient (13). These changes to the LPS structure have been shown to result in larger downstream changes to the lipid and protein composition of the bacterial outer membrane and secreted vesicles (25, 26). OMVs derived from LPS deficient *Neisseria meningitdis* are known to be enriched in many proteins that are absent from the cellular outer membrane (27). A more common modification, such as loss of the O antigen, is sufficient to alter the selection of proteins for inclusion in OMVs. For example, the immunodominant, outer membrane proteins RagA/B of *P. gingivalis* are normally absent in wild-type OMVs, but have been identified in OMVs lacking the O antigen (26). Therefore the loss of the O antigen may influence the role of OMVs as both virulence factor and a trigger of host inflammatory responses.

The present study investigates the composition of both the outer membrane and outer membrane vesicles in *K. pneumoniae* 43816 and an isogenic mutant in *wbb*O using a comprehensive proteomics-based approach. The *wbbO* gene codes for the galactosyltransferase responsible for the synthesis and attachment of the O antigen to the core polysaccharide (10). We hypothesized that a major LPS modification, such as loss of O antigen, would result in distinct changes to both the outer membrane and OMVs proteomic profiles. Purified outer membranes and OMVs were analyzed by LC-MS/MS generating a dataset of over 1,400 proteins identified with high confidence (False Discovery Rate $\leq$ 1%). It was expected that the protein profiles of the OMVs would differ from the source outer membrane. As anticipated many proteins were only identified in either the outer membrane or OMVs. When comparing the wild-type to O antigen-deficient OMVs, there were numerous proteins that were identified in only the wild-type or O antigen-deficient OMVs. To further assess these differences, a smaller dataset was then used to more closely analyze the most abundant proteins found in both the secreted vesicles and the source outer membrane. We also found that outer membrane proteins and other proteins involved in stabilizing or building the cell wall were enriched in OMVs, as compared to the source outer membrane for both wild-type and O antigen-deficient *K. pneumoniae*. It was also anticipated that the absence of the O antigen would influence protein sorting into the OMVs. O antigen-deficient vesicles exhibited a decrease of outer membrane proteins compared to wild-type vesicles, but were enriched in protein chaperones. Based on these findings, we concluded that the protein content of OMVs differs from that of the outer membrane, and that modification of the LPS structure can significantly impact the selective sorting of proteins into OMVs.

Materials and Methods

Bacterial strains and culture conditions

Klebsiella pneumoniae type strain ATCC 43816 and *Klebsiella pneumoniae* Δ*wbb*O, an O antigen-deficient isogenic mutant of *K. pneumoniae* 43816, were used in this study. *K. pneumoniae* Δ*wbb*O was provided by the Younger lab, University of Michigan (10). *K. pneumoniae* strains were cultured in Luria-Bertani (LB) broth, supplemented with 50μg/ml kanamycin for the deletion strain, and incubated at 37℃. Growth curves were performed by inoculating 1:1000 of overnight cultures into 50 mL of LB broth and the absorbance at 600nm (OD_{600}) was measured at thirty minute intervals.

Outer membrane vesicle purification

Outer membrane vesicles (OMVs) were purified as previously described (28). Briefly, 6 liter cultures of each strain were grown to mid-late log phase (OD_{600} 1.5-2.0). Cell free supernatants were obtained by differential centrifugation (15 min at 10,000 × g, then 15 min at 13,000× g) and filtration through a 0.65μm PVDF membrane filter (Millipore, Billerica, MA). The vesicle-containing supernatants were then concentrated through a tangential filtration concentrator unit (Pall Life Science, Ann Arbor, MI) and collected on 100kDa Pellicon filtration cassettes (Millipore). Vesicles were then further concentrated in 100kDa MWCO Amicon concentrators and reconstituted in 60% Opti-Prep (45% final concentration) (Sigma-Aldrich, St. Loius, MO). Vesicles were purified using density gradient centrifugation (OptiPrep/HEPES layers of 40, 35, 30, 25, and 20%). After ultracentrifugation (ON at 100,000 × g), 1 ml fractions were collected, analyzed for vesicle protein content by 12% SDS-PAGE, and visualized with

SYPRO Ruby Red stain. Pure vesicles were pooled, dialyzed overnight in phosphate buffered saline, and concentrated to a final volume of 1 ml.

Relative outer membrane vesicle quantification

Relative OMV production was assessed by a previously described phospholipid assay (42). Briefly, cell free supernatants were collected by centrifugation at 10,000 $\times g$ and filtered through a 0.45 μm membrane. Vesicles were then pelleted by high speed centrifugation (40,000 x g for 2hr), washed and resuspended in MV buffer (50 mM Tris, 5 mM NaCl, 1 mM $MgSO_4$, pH 7.4). An equal volume of chloroform was added and the vesicles were centrifuged at 6,000 $\times g$ for 5 minutes. The bottom, organic layer was then collected and treated with an equal volume of ammonium ferrothiocyanate solution (27.03 g/L $FeCl_3 6H_2O$, 30.4 g/L NH_4SCN). The sample was centrifuged again; the bottom layer was collected, and dried under nitrogen gas. Dried samples were resuspended in 1mL of chloroform and the absorbance at 470nm was measured. Absorbance values were normalized to the OD_{600} of the originating cultures.

Outer membrane protein isolation

Cell fractionation was adapted from a previously described protocol (43). Briefly, cell pellets from 100mL of mid-late log phase cultures were resuspended in Tris-Sucrose solution (20mM Tris, 20% sucrose w/v, pH 8.0), and then lysed with lysozyme (40μL lysozyme/g of cells) and 0.1M EDTA (0.2mL of EDTA/g of cells). The mixture was incubated on ice and 0.5M $MgCl_2$ was added to the lysate. The lysate was centrifuged at 12,000 $\times g$ for 20 minutes, supernatants containing periplasmic proteins was removed, and pellets containing the membrane fractions were resuspended in ice cold Tris (10mM, pH 8.0). Membrane fractions were sonicated

on ice, cellular debris was pelleted (9,000× g, 5 minutes) and the supernatant containing the membranes were then further centrifuged (40,000 × g, 60 min) to remove cytoplasmic proteins. The membrane fractions within the pellet were washed with ice cold Tris, re-suspended in DI H_2O, and stored at -80℃ Membrane fractions were treated with 1.2mL of sarkosyl solution (1.7% sarkosyl w/v, 11 mM Tris), for 20 minutes, and centrifuged at 40,000 × g for 90 minutes to separate the inner and outer membrane fractions. The pellet containing only the outer membrane fraction was washed in ice cold Tris and dialyzed overnight in PBS at 4℃.

Proteomic sample preparation, digestion, and LC-MS/MS analysis

Triplicate samples of both the outer membrane and OMVs from *K. pneumoniae* 43816 and Δ*wbb*O were dissolved in 6 M urea, 50 mM ammonium bicarbonate, 10 mM DTT, and 2% SDS. Fifty μg of total protein was alkylated by addition of iodoacetamide to 10 mM and incubation in the dark for 30 min before trypsin digestion using the FASP method (44). Desalting of tryptic peptides was performed on C_{18} MacroSpin solid-phase extraction (SPE) spin columns (The Nest Group, Inc., Southborough, MA, USA). Samples were lyophilized by centrifugation under vacuum and reconstituted in 0.1% formic acid in high-performance liquid chromatography (HPLC)-grade water. Five μg total protein was loaded onto a 150 μm ID × 2.5 cm capillary trap column prior to separation by reverse-phase HPLC on a 75 μm ID × 10 cm analytical column – both packed with ProteoPep II C_{18} (New Objective, Woburn, MA, USA). The mobile phases consisted of 0.1% formic acid in water for A and 0.1% formic acid in acetonitrile for B. Samples were analyzed by linear gradient elution of 2% - 40% solvent B over 240 minutes. Full MS scans were performed at normal resolution on a LTQ XL (Thermo Scientific, San Jose, CA, USA) with a target of 4×10^4 ions, two microscans, and a 50 ms maximum injection time. MS/MS scans were performed on the top-ten most intense ions with an

ion-traptarget of 5×10^3 ions and dynamic exclusion set for 180 s with one repeat and a limit of 500.

Analysis of mass spectral data

Raw LC-MS/MS data from *K. pneumoniae* 43816 and Δ*wbb*O outer membrane fractions and outer membrane vesicles were searched against a *K. pneumoniae* subset of the European Bioinformatics Institute's (EBI) universal protein resource database (UniProt, 5035 entries) using MASCOT (Matrix Science, London, UK; version 2.2.06). Peptide and protein identifications were validated on the Scaffold platform (version Scaffold_4.3.2, Proteome Software Inc., Portland, OR). Peptide identifications were accepted if greater than 95.0% probability as calculated by the Peptide Prophet algorithm (45), while proteins were accepted at probabilities greater than 95% as calculated by the Protein Prophet algorithm (46). Proteins containing similar peptides were parsimoniously grouped. These parameters resulted in peptide and protein confidences at a false discovery rate (FDR) of less than 1.0% . A smaller dataset consisting of the top 200 most abundant proteins across all samples was used for comparative proteomics analyses. Individual sample classifications were limited to proteins that were identified in at least two replicates. Proteins in each sample were categorized by their cellular localization and biological function. Spectral sums and percentages were used to assess relative abundance between samples. Cellular localizations of unclassified proteins were predicted using PSORTb (47) and biological functions were categorized using cluster of orthologous groups (COG) annotation terms (www.ncbi.nlm.nih.gov/COG). Statistical significance of weighted spectra for each sample was evaluated by single factor ANOVA analyses.

Results

Loss of the O antigen does not impact bacterial growth or production of OMVs, but does alter the outer membrane protein profile.

This study was designed to characterize the effects of O antigen loss on the protein composition of the *Klebsiella pneumoniae* outer membrane (OM) and secreted outer membrane vesicles (OMVs). *wbb*O is the galactosyltransferase involved in the attachment of the O antigen to the LPS core (10). Growth curves were performed to evaluate if the inactivation of *wbb*O and resulting absence of the O antigen altered the growth rate of *K. pneumoniae*. Figure 2.1A shows that the deletion of *wbb*O does not alter the growth rate of the bacteria. We then quantified relative OMV production of each strain to determine if O antigen loss impacted this basic physiologic function. OMVs were harvested from overnight cultures, quantified by a colorimetric phospholipid assay, and normalized to the OD_{600} of the originating culture (42). We found that the amounts of OMVs produced by both wild type and O antigen-deficient *K. pneumoniae* were similar (Figure 2.1B). These data demonstrate that O antigen loss does not influence either growth or OMV production.

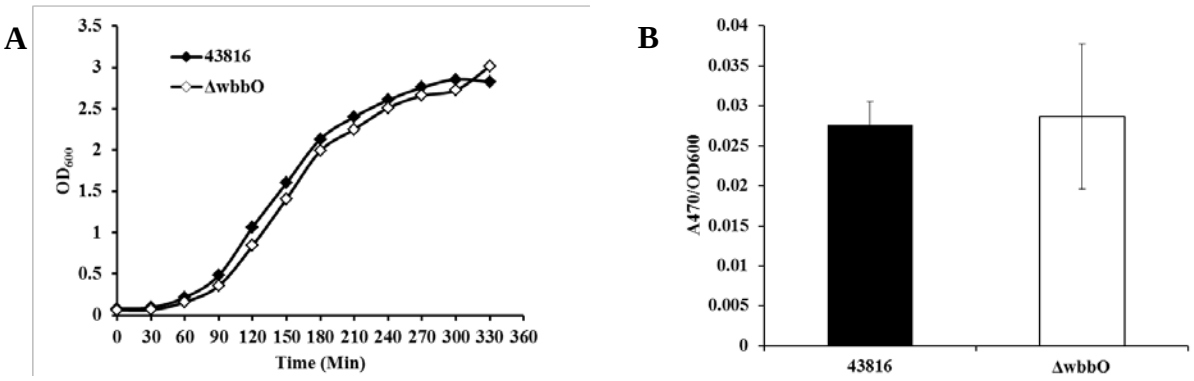

Figure 2.1 The impact of O antigen loss on cellular growth and relative outer membrane vesicle production. (A) Growth curves for *Klebsiella pneumoniae* 43816 and Δ*wbb*O strains cultured in LB broth. (B) Relative OMV production from overnight cultures quantified by phospholipid analysis. Phospholipid content was measured at 470nm and normalized to the OD_{600} of the extracting cultures. Errors bars represent SD.

Because LPS is a major component of both the outer membrane and outer membrane vesicles, we investigated if loss of the O antigen would impact the protein composition of these cellular structures. As seen in Figure 2.2, the protein composition of both the outer membrane and OMVS was altered by loss of the O antigen, resulting in a distinct protein profile in the deletion mutant. Δ*wbb*O OMVs lack many higher molecular weight signals present in both of the OM fractions, as well as the wild type OMVs. Additionally, wild type OMVs have a very prominent signal around 48kDa that is absent in O antigen-deficient OMVs. These observed differences indicate that the absence of the O antigen alters protein expression in both the outer membrane and secreted vesicles. Additionally, the protein profiles of the outer membrane and OMVs from the *wbbO* deletion strain are not identical, indicating that proteins increased in the outer membrane of the deletion mutant are not necessarily packaged into vesicles.

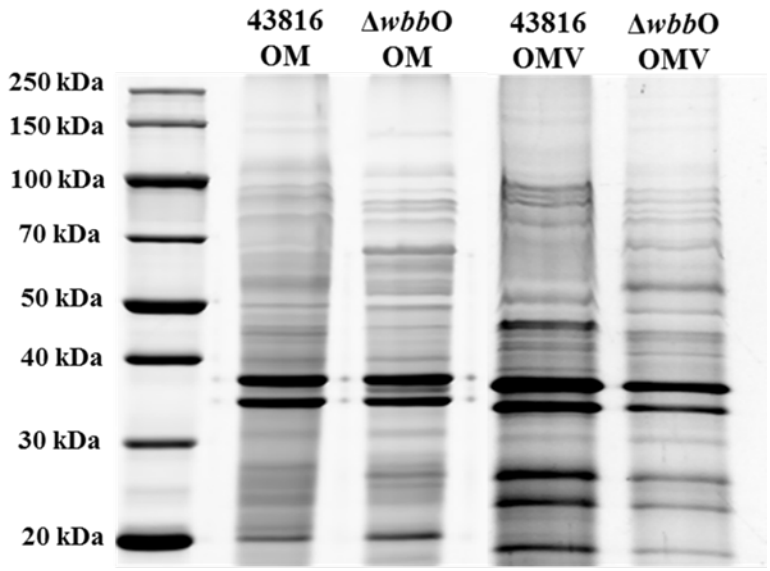

Figure 2.2 O antigen loss influences protein expression in the outer membrane and outer membrane vesicles. Purified outer membranes (OM) and outer membrane vesicles (OMVs) from wild type *K. pneumoniae* and Δ*wbb*O were resolved by 10% SDS-PAGE and visualized by SYPRO Ruby stain.

Quantitative assessment of outer membrane and outer membrane vesicle proteomes

To fully characterize the changes in protein profiles observed in Figure 2.2, we used LC-MS/MS to characterize the OM, and OMV sub-proteomes of *K. pneumoniae* 43816 and the *ΔwbbO* mutant. This generated a dataset of over 1,400 proteins identified with high stringency (< 1.0% FDR). This dataset consisted of 1,334 OM and 1,130 OMV proteins. When comparing the wild type and the deletion strains, 1,057 and 649 proteins were commonly identified in either the outer membrane or the OMVs (Figure 2.3A & 2.3B). Interestingly, many proteins common to both the OM and OMVs were only identified in either the wild-type or O antigen-deficient *K. pneumoniae*, indicating the profound impact of O antigen loss on the protein composition. As shown in Figure 3B, only 649 of the 1,130 OMV proteins were common to both strains, with many proteins exclusively found in secreted vesicles from the Δ*wbb*O strain (n=284) or the wild type (n=197). When comparing the source outer membrane to their OMVs, many proteins were solely found in the cellular OM of either strain (wild type n=513 or *Δwbbo* n=354) (Figure 2.3C & 2.3D). Likewise, the OMV proteome consists of many proteins that are absent in the source OM in wild type (n=98) and O antigen-deficient (n=157) OMVs. These data further support our hypothesis that the loss of the O antigen dramatically impacts not only the composition of the outer membrane itself, but also has a significant effect on which proteins are selectively packaged into OMVs.

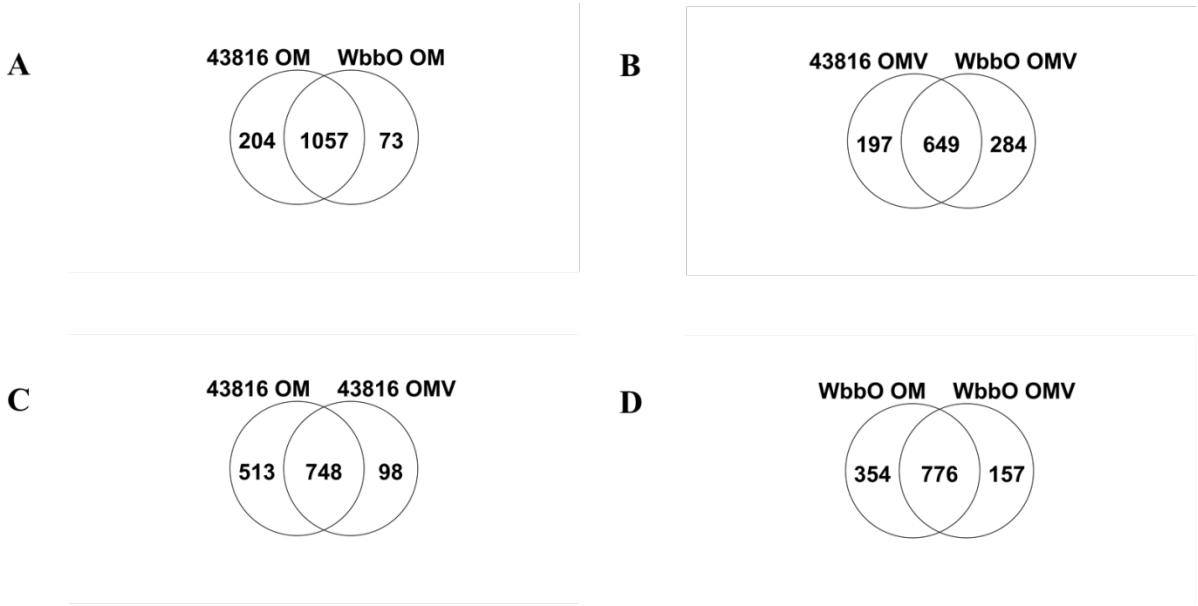

Figure 2.3 Semi-quantitative evaluation of the OM and OMV proteome of *K. pneumoniae* 43816 and Δ*wbb*O. Proteins were evaluated by LC-MS/MS and analyzed in SCAFFOLD Q+S generating a 1,400 protein dataset. Scaffold Q+S software was used to evaluate general quantitative comparisons between the numbers of proteins identified in (A) 43816 and Δ*wbb*O outer membranes, (B) outer membrane vesicles, and outer membrane and outer membrane vesicle quantitative comparisons for *K. pneumoniae* (C) 43816 and (D) Δ*wbb*O.

The O antigen influences packaging of outer membrane proteins into vesicles

This study focused on identifying the most significant changes to the protein composition within the OM and OMVs as a result of O antigen loss. For this more focused analysis, we generated a smaller dataset representing the top 200 proteins with the highest spectral counts across all samples (Table S1). The proteins excluded from this data subset either had very low spectral counts or were proteins that were only identified in one replicate and were not identified in other samples. This dataset was used for both quantitative and qualitative assessments of the cellular localizations and functional classification of these top 200 proteins.

The cellular locations of proteins found in OMVs were first assigned by either their designation in UNIPROT, or predicted using PSORTb (47). Analysis of this subset revealed that outer membrane proteins represented a lower percentage of the total spectra in O antigen-deficient vesicles compared to wild type OMVs (Figure 2.4A). Loss of O antigen resulted in a statistically significant (p=0.014) decrease in the amount of outer membrane proteins in Δ*wbb*O OMVs compared with OMVs from the wild-type strain (Figure 4B). There were no significant differences in outer membrane protein enrichment between the cellular outer membranes. However, when comparing outer membrane proteins from the cellular outer membrane to those in the OMVs, we see a significant difference in the degree of this selective packaging. Across both the wild type and the mutant strains, outer membrane proteins are enriched in OMVs as compared to the source OM (Figure 4B). Vesicles from wild type *K. pneumoniae* demonstrated statistically significant enrichment in outer membrane proteins (p=0.001) compared to the source outer membrane, while Δ*wbb*O outer membrane protein enrichment in OMVs did not (p=.083). These data support the current hypothesis of selective packaging of protein cargo into OMVs,

and demonstrate how this selective packaging process can be altered by loss of the O antigen (18, 25, 30).

Given this significant change in the proportion of outer membrane proteins in vesicles, we then assessed the cellular localization and functional classifications of all proteins from our 200 protein dataset (Table S1). We first categorized OMV proteins by their cellular localization (Figure2. 4A). This analysis confirmed our earlier result that outer membrane vesicles are enriched in outer membrane proteins, and also revealed the presence of many cytoplasmic proteins. Outer membrane (n=25) and cytoplasmic proteins (n=77) represent 46.7% and 45% of the total spectra in vesicles from wild type *K. pneumoniae*. In the Δ*wbb*O strain, the number of outer membrane proteins (n=25) is the same as wild-type. However, the overall abundance of these same proteins is decreased in Δ*wbb*O OMVs where outer membrane proteins only represent 24.8% of the total spectra (Figure 2.4A). Cytoplasmic proteins, many of which can also be found in the periplasm, are more highly enriched in Δ*wbb*O OMVs (n=90) than those of the wild-type strain. Additionally, a small number of inner membrane proteins (n=13; n=9) were identified in both the wild type and Δ*wbb*O OMVs. Many of these inner membrane proteins are peripheral membrane proteins and not directly tethered to the inner membrane itself, allowing for their movement into secreted vesicles. Surprisingly, only 2% and 5.5% of the proteins identified in this smaller dataset were periplasmic proteins in Δ*wbb*O and wild type OMVs, respectively. These data demonstrate that loss of the O antigen has a significant effect upon the protein composition of outer membrane vesicles, resulting in vesicles that contain a greater abundance of cytoplasmic and periplasmic components.

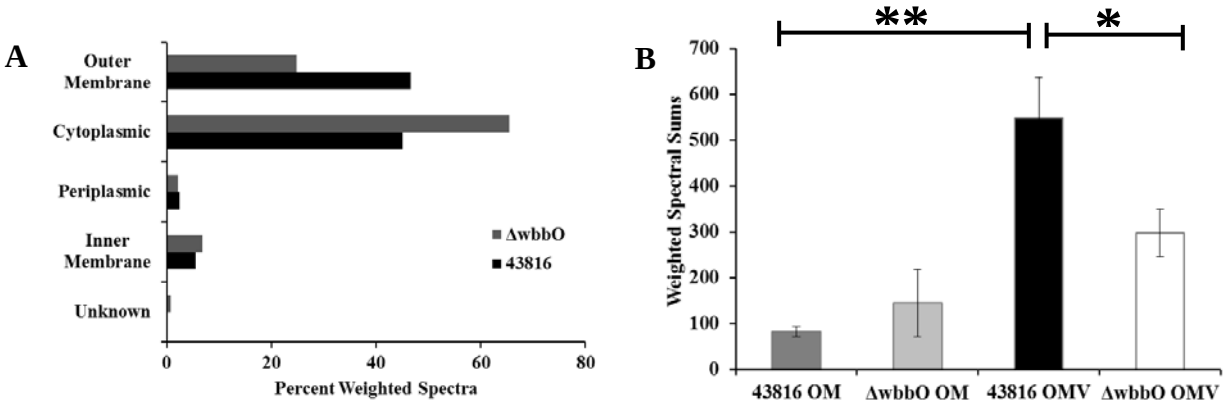

Figure 2.4 Loss of the O antigen alters outer membrane protein composition in outer membrane vesicles. (A) Percent of total spectra by cellular location of *K. pneumoniae* 43816 and Δ*wbb*O outer membrane vesicles. (B) Relative quantification of outer membrane proteins in OM and OMVs from *K. pneumoniae* 43816 and Δ*wbb*O. Statistical significance of weighted spectral sums was assessed by single factor ANOVA (* $p<0.05$, ** $p<0.001$). Significance between groups is represented by horizontal brackets. Error bars represent SD.

Functional classification of proteins in outer membrane vesicles and the cellular outer membrane

The top 200 most abundant spectra were then used to assign COG functional classifications to proteins (www.ncbi.nlm.nih.gov/COG). Table 1 reports the percentage of spectra for each category from either outer membrane or outer membrane vesicles. The percentage of total spectra was used to evaluate the proportional representation of proteins from each category in the respective sample. COG category "M" includes proteins involved in cell wall/membrane/envelope biogenesis which are highly abundant in OMVs from both strains, as well as in the outer membrane of the Δ*wbb*O strain. The majority of the proteins belonging to COG category "M" are localized in the outer membrane (Table S1). The percent spectra of category M further demonstrates enrichment of proteins from this category in OMVs over the outer membrane itself, and less in the OMVs from the Δ*wbb*O strain compared to the wild type (Table S1).

Table 1. COG functional classification of outer membrane vesicle and cellular outer membrane proteins. The percentage of total spectra is given for each COG category.

		Membrane		Vesicles	
	COG Functional Category	**43816**	**Δ*wbb*O**	**43816**	**Δ*wbb*O**
	Cellular Process & Signaling				
D	Cell cycle control, cell division, chromosome partitioning	0.73963	0.49352	0	0.57427
M	Cell wall/membrane/envelope biogenesis	6.64828	11.3234	29.9285	17.3236
O	Post-translation modification, protein turnover, chaperones	12.098	15.4826	8.04133	14.9933
T	Signal Transduction mechanisms	0.22936	0.1326	0	0
U	Intracellular trafficking, secretion, and vesicular transport	0.98403	0.63012	0	0.15401
V	Defense mechanism	0.21232	0.15126	0	0
	Information Storage & Processing				
J	Translation, ribosomal structure and biogenesis	20.7215	24.1436	24.7464	25.6707
K	Transcription	4.31308	3.26736	2.0423	3.4625
	Metabolism				
L	Replication, Recombination and repair	0.50665	0.23835	0.25479	0.98273
C	Energy production and conversion	20.0042	15.2696	9.99936	15.9548
E	Amino acid transport and metabolism	6.3365	3.70062	0.70088	1.08989
F	Nucleotide transport and metabolism	2.0942	1.46976	0	0.72828
G	Carbohydrate metabolism and transport	16.5585	15.8073	4.82551	8.06051
H	Coenzyme transport and metabolism	0.84585	0.73061	1.22301	0.60297
I	Lipid transport and metabolism	0.89372	0.70761	0.48885	0.84451
	Poorly characterized				
P	Inorganic ion transport and metabolism	2.34052	1.91886	0.64597	0.5514
*	Not in COG genes	0.61543	1.27375	12.4129	4.86944
R	General function prediction only	2.5581	2.26888	2.27508	2.27673
S	Function unknown	1.30019	0.99023	2.41547	1.86028

To determine the importance of the changes in these functional categories, single factor ANOVA was used on spectral sums for each COG category to assess statistical significance between samples. As seen in Figure 2.5A, there was a statistically significant difference in the abundance of proteins from COG category "M" between wild type vesicles and the source outer membrane. There is also a near significant (p=0.07), difference between COG category M protein abundance between wild-type and O antigen-deficient OMVs (Table S2).

Many of the COG category "M" proteins that are enriched in OMVs are major outer membrane porins such as OmpA, OmpK35, and OmpK36. Outer membrane proteins not associated with transport, such as murein lipoprotein, SlyB, Tsx, and YfaZ were also significantly enriched ($p<0.05$) in OMVs from both strains (Table S2). In the vesicles derived from the Δ*wbb*O strain, all of the previously described outer membrane proteins are less abundant than in the wild type OMVs. The spectral counts of OmpA, OmpK17, TolC, MipA, YfaZ were significantly lower in OMVs from the Δ*wbb*O strain, indicating that the O antigen influences protein integration into OMVs (Table S2).

Consistent with our observation of increased outer membrane protein enrichment in wild-type vesicles, OmpA, OmpK17, TolC, MipA, and BtuB all had statistically significant increases in spectral counts when comparing vesicles to source membrane in the wild type strain (Tables S1 and S2). Proteins YfaZ and BtuB were almost entirely present in OMVs having very low spectra from the OM fractions, demonstrating the potential for extreme enrichment in vesicles from low concentration in the source outer membrane.

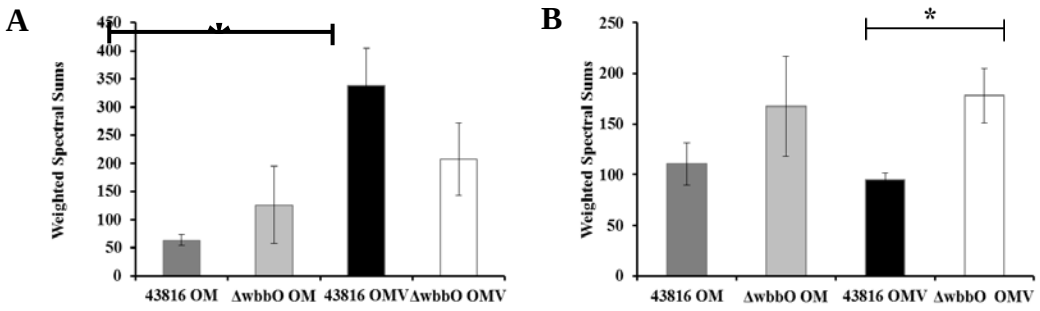

Figure 2.5 Abundance of COG functional category "M" and "O" proteins. Weighted spectral sums of proteins involved in (A) cell wall/membrane/envelope biogenesis (Category "M"), and (B) PTM, protein turnover, and chaperones (Category "O") were averaged between replicates. Statistical significance was based on individual weighted spectral counts and assessed by ANOVA (* = p <0.05). Significance between groups is represented by horizontal brackets. Error bars represent SD.

When comparing the cellular OM fractions, the major outer membrane proteins were slightly enriched in Δ*wbb*O OM samples (Figure 2.4B & 2.5A). One possible explanation for the difference in protein levels between the wild type and the knockout strain is that deletion of *wbb*O results in altered levels of total cellular production of many of these outer membrane proteins. In order to confirm that the Δ*wbb*O mutation is not altering outer membrane protein transcription through a secondary mechanism of gene regulation, RNA was extracted from mid-late log phase cells to quantify relative outer membrane protein transcript levels (OmpA, OmpK35, and OmpK36) via qPCR. *K. pneumoniae* Δ*wbb*O outer membrane protein transcript levels were slightly, but not significantly, down-regulated compared to wild type *K. pneumoniae* (data not shown). These data indicate that deletion of the *wbb*O gene is not altering outer membrane protein gene expression to the degree that is observed in our proteomic analysis.

O antigen-deficient OMVs are enriched in chaperones

K. pneumoniae Δ*wbb*O OMVs exhibit decreased outer membrane protein cargo and increased numbers of proteins involved in post-translational modification, protein turnover, and chaperones (COG category "O") (Figure 2.5B). Many of these proteins are chaperones involved in protein turnover of the cellular envelope (Table S1). In particular, GroEL, a cytoplasmic protein known to be associated with the outer membrane of *K. pneumoniae* and other Gram-negative bacteria (48-50), was the most prominent protein found in Δ*wbb*O OMVs, and was the third most abundant in wild-type OMVs. When compared to wild type *K. pneumoniae*, GroEL levels in the Δ*wbb*O strain were highly enriched in both the OM and OMVs (Figure 2.6A & Table S2). Other chaperones, such as the cytoplasmic and periplasmic proteins DnaK and DegP,

were also enriched in Δ*wbb*O OMVs. DnaK enrichment was only observed in the OMVs derived from Δ*wbb*O (Figure 2.6B), while GroEL and DegP exhibit enrichment in vesicles from both strains. DegP was highly enriched in OMVs from both strains, and the level of DegP enrichment in Δ*wbb*O OMVs was statistically greater than DegP levels in Δ*wbb*O OM (Figure 2.6C). This indicates that chaperone production and packaging into vesicles may be a compensatory role for the lack of outer membrane proteins expressed in Δ*wbb*O OMVs.

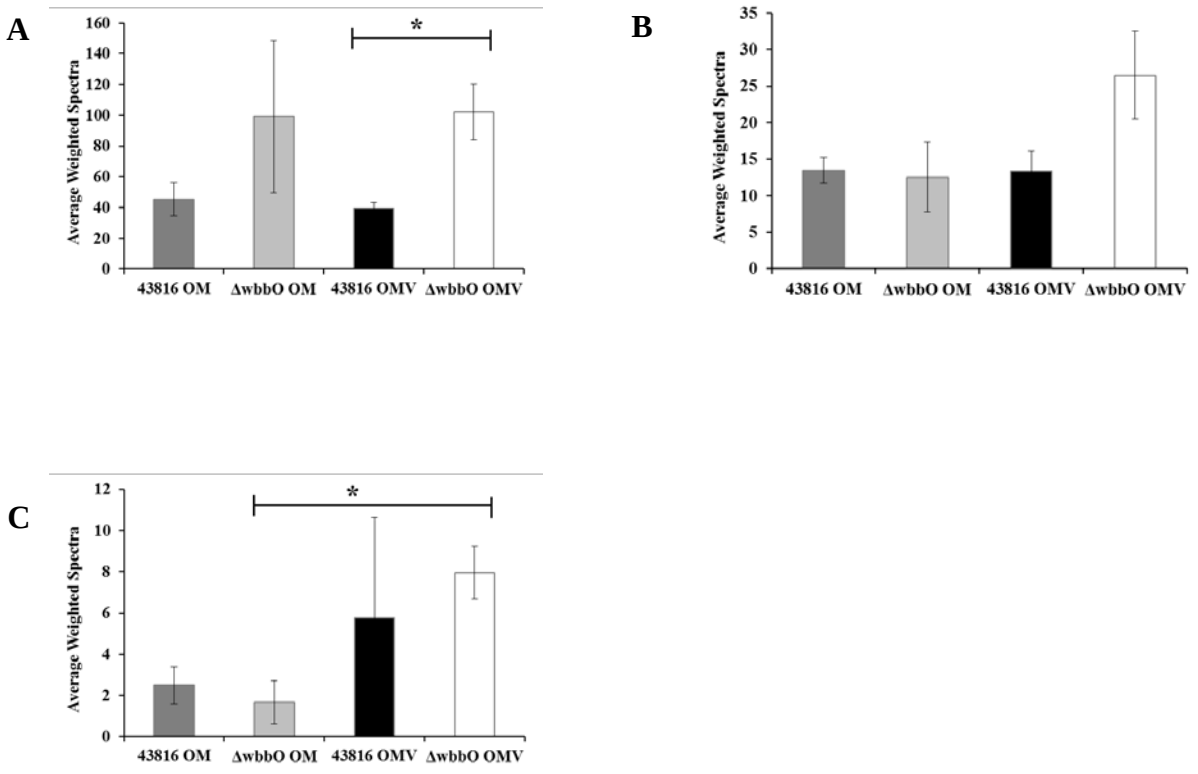

Figure 2.6 Chaperone enrichment in *K. pneumoniae* Δ*wbb*O outer membrane vesicles**.** Weighted spectra of the chaperones (A) GroEL, (B) DnaK, and (C) DegP were averaged between replicates. Statistical significance was based on individual weighted spectral counts and assessed by ANOVA (* = p <0.05). Significance between groups is represented by horizontal brackets. Errors bars represent SD.

Discussion

The goal of this study was to compare and characterize the sub-proteomes from both the outer membrane and secreted outer membrane vesicle (OMVs) of *Klebsiella pneumoniae*. Additionally, we characterized how a single mutation, resulting in the loss of LPS O antigen impacted the packaging of protein cargo into outer membrane vesicles. This comprehensive proteomic study is the first to directly compare the outer membrane and OMV sub-proteomes in *K. pneumoniae*. Our findings support the hypothesis that a selective sorting process is utilized to direct packaging of protein cargo from the outer membrane into OMVs. This sorting process is altered by O antigen loss, with fewer outer membrane proteins, but more chaperones, packaged into O antigen-deficient OMVs. Together, the proteomic analyses presented in this study demonstrates that LPS modification impacts the process of packaging cargo into secreted vesicles.

We initially hypothesized that such a major modification to the structure of LPS as O antigen loss would have a significant effect on either the rate of bacterial growth or the rate of outer membrane vesicle production. However, this was not the case, as neither the growth rate nor the rate of OMV production was significantly impacted by this isogenic mutation. Other studies targeting the *wbbO* gene in *Klebsiella* have also observed no change in growth rate (3). O antigen-deficient *Pseudomonas aeruginosa* (ΔWbpM) was reported to a have lower stationary phase cell density in the O antigen mutant, however, this could be attributed to the inactivation of the nucleotide sugar epimerase/dehyratase, *wbp*M, rather than loss of the *wbb*O galactosyltransferase (25).

In agreement with previous findings, the absence of the O antigen was not observed to alter OMV production (25, 26). However, while the loss of the O antigen does not affect these basic physiological processes, alterations to the protein composition of both the outer membrane and secreted outer membrane vesicles were observed in our experiments. These data demonstrate how the deletion of a single gene in the LPS synthesis pathway can result in large-scale reorganization and compensatory changes to the outer membrane.

The total protein composition of both the outer membrane and naturally secreted outer membrane vesicles were evaluated by LC-MS/MS analysis. In order to have a highly comprehensive analysis, we utilized the FASP method to process the total protein content of both the outer membrane and OMVs. This method allowed us to also identify lower abundance proteins that are often omitted when excising proteins from gels. This analysis resulted in an initial protein dataset of 1,400 proteins identified with high confidence, which greatly expands the known outer membrane vesicle proteome of *Klebsiella pneumoniae*. To date, there has only been one other proteomic study conducted on *K. pneumoniae* OMVs. Although the study by Lee et al. (2012), demonstrated that *K. pneumoniae* OMVs elicit the murine innate immune response, the proteomic analysis of this study was limited to excised samples from a protein gel, resulting in a small dataset of only 159 proteins (30). Even with this limited dataset, a number of proteins, including MipA, Lpp, and DegP were found in common with our data. Interestingly, Lee et al. (2012), reported that OmpX, or OmpK17, was the single most abundant outer membrane protein in their OMVs, whereas we identified OmpA as being the most abundant. Beyond the differences in methodology, this discrepancy could be attributed to strain specific variations in *K. pneumoniae,* or the possibility that the OMVs were harvested from different stages in bacterial growth in the two studies.

An initial evaluation of our large dataset revealed that many proteins were present in higher concentrations in either the outer membrane or secreted vesicles. The protein profiles of OMVs are known to be distinct from those of the outer membrane, and a selective sorting mechanism has been suggested to be involved in OMV formation (25-27). Initial quantitation of this large dataset confirmed that the protein profiles of the OMVs in *K. pneumoniae* are distinct from those of the outer membrane, supporting this current hypothesis. To evaluate the differences in the outer membrane and OMV sub-proteomes in more detail, we utilized a smaller dataset of the top 200 proteins across all samples. This more detailed analysis revealed that, like many other bacterial species, wild-type OMVs from *K. pneumoniae* are highly enriched in outer membrane proteins, while cytoplasmic and periplasmic proteins represent a smaller proportion of OMV cargo (18, 25, 26, 30). In our O antigen mutant, we see a dramatic increase in OMV cargo originating in the cytoplasm as compared to wild-type OMVs. This alteration of the relative content indicates that the O antigen does contribute to the process of cargo sorting and selection into OMVs.

The majority of the proposed mechanisms for the production of outer membrane vesicles focus on the actions of proteins from COG category “M”. The enrichment of proteins from this functional category is common to OMVs from many Gram-negative bacterial species (18, 39, 51, 52). In particular, Lpp and OmpA, which both act to create stabilizing linkages between the outer membrane and the peptidoglycan layer, are thought to be critical regulators of this process (53). Deletion mutants of either of these two proteins result in a hyper-vesiculation phenotype (54-56), while down-regulation of OmpA by the small RNA *VrrA* has been associated with increased vesicle production in *Vibrio cholera* (57).

OmpA was the most abundant outer membrane protein identified in all samples and was highly enriched in OMVs. However, the enrichment of proteins such as OmpA, TolC, Lpp, and MipA, was much less pronounced in the O antigen-deficient strain of *K. pneumoniae*. The pronounced level of OmpA in all samples, and the lack of change in the rate of vesicle production indicate that the loss of O antigen does not directly impact the rate of vesicle production, but may instead impact the selection of cargo to be packaged into vesicles.

There have been discrepancies regarding whether cytoplasmic proteins are normal constituents in the lumen of OMVs or an artifact of cellular lysis. Much of this discussion has been based on the results of early studies of bacterial vesicles that utilized harsh treatments to artificially generate outer membrane liposomes, or relied on less rigorous purification protocols. Studies such as this one, which follow standardized purification methods, utilizing density gradients to remove cellular debris, have confirmed that some cytoplasmic contents are normal components of outer membrane vesicles (37, 58, 59). Most of the cytoplasmic proteins found in OMVs in this study, including GroEL (Hsp-60) and EfTu, are commonly seen in vesicles purified from bacterial species. Additionally, many of these proteins have more recently been observed on the bacterial outer surface, indicating that trafficking of proteins from the cytoplasm to the outer surface may be a component of vesicle formation (60).

Deletion of the *wbbO* galactosyltransferase resulted in large scale alterations to the composition of the outer membrane beyond just the truncation of LPS polysaccharides. The protein composition of both the outer membrane and secreted vesicles was significantly altered by this single deletion. Even with this alteration, many outer membrane proteins remained enriched in Δ*wbbO* OMVs, with 25 outer membrane proteins common to both wild-type and O antigen-deficient vesicles. However, the spectral counts for outer membrane proteins were

significantly less in Δ*wbbO* OMVs than the wild type. Outer membrane proteins represented approximately 25% of proteins identified in Δ*wbbO* OMVs, as compared to 46.7% representation in wild-type OMVs. In fact, while enrichment of outer membrane proteins from the OM to OMVs was still apparent in the mutant strain, this decrease in the level of enrichment resulted in the OMVs no longer being statistically different from their source membrane. This change in the outer membrane protein content upon alteration of LPS is consistent with studies of other mutants, such as the deletion of the *wbpM* epimerase/dehydratase in *Pseudomonas* (25) and LPS deficient *Neisseria* (27). Together these studies all indicate that the structure of LPS directly impacts the selection of protein cargo during vesicle formation.

Haurat et al. (2011), completed a similar study investigating the packaging of gingipains into the outer membrane vesicles of *P. gingivalis*. In contrast to the present and other data, they found that deletion of either the *porS* flippase, or the *waaL* ligase, did not significantly impact outer membrane protein composition, but did alter cargo selection for vesicles (26). *P. gingivalis* normally contains two varieties of LPS, O and A chain, with one of these natural variants lacking the O antigen entirely. This key difference in membrane lipid content can explain how the protein content of the *P. gingivalis* outer membrane is not greatly altered, unlike the results seen in our study. The more modest changes seen in the *P. gingivalis* membrane may reflect the moderating effect of altering some, but not all, LPS in the bacterial cell.

Even with these more modest changes to the source membrane, Haurat et al. (2011), did demonstrate significant changes to OMV protein cargo, and proposed a mechanism for sorting and packaging of proteins into OMVs that requires direct interaction between the O antigen sugars and the proteins to be packaged (23, 26). Our data supports the hypothesis that O-antigen plays an important role in protein sorting into OMVs. However, our data also indicate that this is

not the sole mechanism of sorting; as enrichment of outer membrane proteins also occurs in our O antigen-deficient strain, albeit to a reduced degree. We confirmed via Real-Time PCR that expression of the major outer membrane proteins is not impacted by O antigen loss, and levels of these proteins are similar in the source outer membranes of both the wild-type and deletion strains. Therefore, this reduction of OMP content in secreted vesicles is not due to reduced expression of these proteins, nor reduced content in the source membrane.

The previously mentioned model has been proposed for bacterial species that express A band (neutral) and B-band (charged) LPS, such as *P. aeruginosa* and *P. gingivalis* (25, 26). It is hypothesized that charged O antigen residues may directly affect the integration of certain protein cargo into OMVs (23, 29). Proteins containing a yet-to-be identified microdomain may have a strong affinity for the charged O antigen, leading them to directly interact with the O antigen, resulting in packaging as protein cargo during the budding and release of OMVs from the membrane. However, this model is limited to bacterial species with known charged LPS chemotypes. In most enteric bacteria, the phosphates of the inner core, rather than the O antigen, are responsible for the net negative charge of the LPS molecule (61). However, in *K. pneumoniae*, the core oligosaccharide differs from other Enteric's because it lacks these charged phosphates, and instead contains GalU residues that are responsible for the net negative charge. These variations in molecular structure would have a direct impact on protein sorting into OMVs using the current proposed model. Further, it has been reported that *E. coli*, *Salmonella*, and *Klebsiella* deep rough mutants, which lack the core phosphates or GalU residues, had decreased outer membrane protein content indicating that the core of the LPS molecule may be involved in the folding and integration of OMPs into the membrane (61-66). Finally, secondary sorting factors have been further hypothesized to interact with both the LPS O antigen and cargo

proteins resulting in selective sorting into OMVs (23, 29). Our data support the idea of the LPS molecule being critically involved in the process of cargo sorting, but not being the sole player in this function.

The loss of the O antigen resulted in OMVs with reduced levels of proteins from COG category "M", but were significantly enriched in COG category "O" proteins involved in post-translational modification, protein turnover, and chaperones; whether periplasmic or cytoplasmic. GroEL, a cytoplasmic chaperone also known to be a surface associated protein, was the most abundant protein identified in both the outer membrane and OMV of the Δ*wbbO* strain, and had been identified as a significant component of vesicles from *A. baumannii* and *F. novicida* (67, 68). While GroEL has classically been considered a cytoplasmic chaperone, there is an increasing amount of data demonstrating that this protein can be associated with the outer surface of both Gram-positive and Gram-negative bacterial species (40, 48, 49). Cytoplasmic GroEL functions as a chaperone that modulates protein folding; but surface associated GroEL has been shown to promote host inflammation and phagocytosis of several bacterial species (48, 50, 69). GroEL expression is known to increase in response to environmental stress and the significant enrichment of this protein in Δ*wbbO* OMVs could likely be a compensatory role for physiologic stress as a result of LPS modification.

The accumulation of mis-folded proteins and proteinaceous waste in the periplasm has been well documented to trigger the envelope stress response and increase formation of OMVs (70). It is therefore unusual that the Δ*wbbO* strain did not exhibit increased vesicle formation, but rather had vesicles with increased chaperones and proteases, such as DnaK and DegP. Additionally, we observed decreased levels of outer membrane proteins in vesicles, but not the membrane. It is unclear whether these outer membrane proteins are not being packaged into

vesicles of the mutant strains, or if they are being degraded by the more plentiful chaperones and proteases. The enrichment of these chaperones in Δ*wbbO* outer membrane vesicles may serve to remove mis-folded outer membrane proteins that otherwise would be removed via OMVs. This hypothesis would explain why we do not observe a hyper-vesiculating phenotype, as periplasmic turgor pressure could be relieved by degradation of proteins within the periplasm itself.

In particular, DegP, a dual function periplasmic chaperone and temperature-dependent protease, may play a significant role in this compensatory response (53, 71). DegP mutants have been well established to produce hyper-vesiculating phenotypes (70, 72). In the current study we found that DegP was enriched in OMVs from both the wild-type and mutant strains; and was more abundant in O antigen-deficient OMVs. Increased DegP content may in fact represent a compensatory mechanism to adjust for the inability of outer membrane proteins to be properly sorted into OMVs for secretion out of the cell. The degradation of OMPs by DegP would explain why the wbbO mutant does not produce a hyper-vesiculating phenotype. Further studies focusing on the interactive effects of mutants in *degP* or other proteases and *wbbO* together are needed to clarify the role that each plays in controlling OMV cargo selection and formation.

In conclusion, this is the first comprehensive study analyzing both the outer membrane and OMV sub-proteomes in *Klebsiella pneumoniae*. This study provides strong support to the existence of a selective sorting mechanism for the integration of proteins into OMVs. This study also demonstrated how the absence of the O antigen from the cell surface impacts this selective sorting mechanism. While our data indicate that the carbohydrate portion of the LPS molecule may play a significant role in proteins sorting for OMVs across species, it also demonstrates how molecular variations to this structure may impact the sorting process. The decreased presence of OMPs, and increased presence of proteases such as DegP, in mutant OMVs indicate that

physiologic adaptations other than increased OMV production may occur in response to the loss of the O antigen. Future studies will focus on determining how the mechanisms of cargo sorting and envelope stress responses interact to impact both OMV production and composition.

Chapter 3

Inflammatory Responses Elicited by the Cellular Outer Membrane and Secreted Outer Membrane Vesicles Derived from Wild Type and O antigen-Deficient *Klebsiella pneumoniae*

Introduction

Gram-negative bacteria naturally release outer membrane vesicles (OMVs) during all stages of cellular growth (18). OMVs are 20-200nm spherical bilayers released from the outer membrane and therefore contain outer membrane components such as lipopolysaccharide (LPS), phospholipids, and outer membrane proteins as well as periplasmic and cytoplasmic proteins within their lumen (19). The production of OMVs removes over-expressed or mis-folded proteins to relieve bacterial envelope turgor pressure (19). Toxic compounds such as attacking phages or host antimicrobial peptides can be removed from the bacterial cell through the release of OMVs (20). Vesicles are also involved in cell-to-cell communication and have been known to be involved in the transfer of antibiotic resistance enzymes and horizontal gene transfer between bacterial species (21, 22).

Common components of OMVs are pathogen associated molecular patterns (PAMPs) such as LPS and outer membrane proteins that stimulate host immune responses. LPS is important for the maintenance and integrity of the outer membrane and contains molecular structures that are recognized by the immune system (6). LPS is a three part structure consisting

of the lipid A, core oligosaccharides, and the O antigen. The lipid A portion of the molecule is integrated into the bacterial outer membrane and has a structure that is conserved among Gram-negative, enteric bacteria (6). Lipid A acts as a PAMP for toll-like receptor 4 (TLR-4), the pattern recognition receptor on the surface of phagocytic cells. The interaction between LPS and the TLR-4-CD14-MD2 complex results in the activation of NF-κB and the production of inflammatory cytokines (8, 9).

Although LPS is the Gram-negative endotoxin responsible for activation of the inflammatory response, LPS modifications are commonly observed in pathogenic bacteria. For example, *E. coli* and *Salmonella thyphimurium*, can exhibit mutations in the genes responsible for the synthesis and attachment of the O antigen resulting in a rough LPS phenotypes (12). Cystic fibrosis patients have been observed with O antigen-deficient strains of *P. aeruginosa (14)*. *Yersinia pestis*, causative agent of the plague, is an exclusively O antigen-deficient bacterium (15). Other pathogens, such as strains of *Acinetobacter baumannii* and *Neisseria meningitis*, have been isolated lacking LPS entirely (16, 17).

Modifications to the LPS endotoxin, such as O antigen loss, alter host immune responses. It has been demonstrated both *in vitro* and *in vivo* that pathogens displaying a rough LPS phenotype have altered immune responses compared to bacteria containing smooth LPS (32-35). Smooth LPS requires LPS binding protein and the CD14 receptor to activate the production of inflammatory cytokines. Rough LPS, lacking an O antigen, can initiate the production of inflammatory cytokines independently of LPS binding protein or the CD14 receptor by only

interacting with TLR-4 and MD-2 (33, 35). These studies demonstrated that the absence of the O antigen alters host immune responses. However, the previously described studies used chemically extracted LPS and it has been shown that vesicle-bound LPS and protein cargo produced a significantly stronger inflammatory response when compared to purified LPS (31, 73).

Because OMVs are highly enriched in LPS, modifications to the LPS would also impact the immune response to OMVs. Vesiculation is the main LPS delivery mechanism that Gram-negative pathogens employ during an infection, however, LPS is not the sole molecule responsible for activating the inflammatory response. OMVs are also enriched in other bacterial PAMPs such as outer membrane proteins that along with LPS, synergistically activate the OMV mediated immune response (31, 74). LPS modifications also cause variation to the protein components of the outer membrane. It has also been demonstrated in *P. aeruginosa* and *P. gingivalis* that the absence of the O antigen alters the protein cargo found in OMVs (25, 26). Therefore O antigen loss and resulting changes to the protein content of the membrane could drastically alter the inflammatory response to OMVs.

OMVs are formed and released from the cellular outer membrane suggesting that the OMV content is reflective of the outer membrane (18, 23). Although the OMVs are derived from the cellular outer membrane, the protein content of OMVs is not identical to the protein content of the cell. In the previous chapter, we demonstrated that OMV protein profiles are distinct from those of the outer membrane in *K. pneumoniae*. Specifically we demonstrated that outer membrane proteins involved in building and stabilizing the cell envelope were highly enriched in OMVs compared to the cellular membrane. The previously described proteomic study also provided support that the loss of the O antigen alters the protein cargo of OMVs. We found that

O antigen-deficient OMVs were less abundant in outer membrane proteins compared to wild type OMVs, but were highly enriched in chaperones.

LPS modifications and OMV production occur simultaneously during infections and should be evaluated together to understand the pathogenicity associated with *K. pneumoniae*. Currently there is only one study evaluating the immune response to *K. pneumoniae* OMVs, however, this study did not include a side-by-side analysis of the OM and OMV immune responses (30). The purpose of the current study was to compare the inflammatory responses elicited in macrophages treated with cellular outer membrane fractions or purified OMVs from *K. pneumoniae* 43816 or an isogenic mutant in *wbbO* lacking its O antigen. Because OMVs are highly enriched in outer membrane proteins which are known to enhance the inflammatory response, it is predicted that the OMVs will produce a much more potent inflammatory response than the cellular outer membrane. It is also anticipated that O antigen-deficient *K. pneumoniae* OM and OMVs will produce distinct inflammatory responses compared to the wild type strain. O antigen-deficient OMVs contained a reduced amount of outer membrane proteins compared wild OMVs. Interestingly OMVs lacking their O antigen were highly enriched in chaperones, which are also known to stimulate the inflammatory response, making it difficult to predict whether the loss of the O antigen will strengthen or dampen the inflammatory response.

Materials and Methods

Bacterial Culture Conditions, Purification of Outer Membrane Vesicles and Outer Membrane Fractions

K. pneumoniae strains 43816 and Δ*wbbO* growth conditions, and OMV and outer membrane fraction purification protocols were previously described in chapter 2.

Macrophage Culture and assay set up

RAW246.7 or MH-S murine macrophages were grown in RPM1 media supplemented with 10% fetal bovine sera and penicillin-streptomycin-amphotericin B. Cells were grown at 37℃ with 5% CO_2 for all experiments. Confluent RAW 246.7 cells were seeded into a 96 well plate (1 x10^5 cells per well) and treated with either wild type or O antigen-deficient OMVs or OM fractions at varying concentrations based on protein content (0.01 μg/ml, 0.001 μg/ml, 0.0001 μg/ml) for 3 hours. Untreated macrophages were used for the control group.

Macrophage Responses and Inflammatory Cytokine Production

Total RNA was collected from macrophages using the RNeasy Kit and purified with a DNase treatment to remove any residual DNA (Qiagen, Valencia, CA). cDNA was synthesized from 1 μg of mRNA using an oligo(dT)16 primer (Operon, Huntsville, AL) and Protoscript II according to the manufacturers protocol (New England bioscience). PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). Real-Time PCR assays were performed using LuminoCt SYBR® Green qPCR ReadyMix on a Eppendorf RealPlex2 unit. Cytokine gene expression was normalized to actin expression and relative gene expression was quantified using the ΔΔCT method (24). To confirm that the mature cytokine gene products are being secreted by macrophages, the culture supernatants were collected and cytokine protein production was quantified by enzyme linked immunosorbent assay (ELISA, R&D Systems/BD) according to the manufacturer's protocol. Statistical comparisons between groups will be evaluated using the single-factor analysis of variance, followed by Tukey's post hoc test ($n \geq 4$; $p \leq 0.05$).

Results

Proteomic analysis of outer membrane (OM) and outer membrane vesicles (OMVs) derived from wild type and O antigen-deficient *Klebsiella pneumoniae* revealed that the OM and OMV sub-proteomes are distinct from one another. In chapter two, we sought to evaluate if the distinct protein profiles observed between the OM and OMVs would result in different inflammatory responses. RAW 264.7 murine macrophages were treated with sequential dilutions of either purified OMVs or OM fractions isolated from wild type (43816) and an O antigen mutant (Δ*wbbO*) *K. pneumoniae*. After a 3 hour incubation, gene expression and cytokine protein secretion were assessed. Relative gene expression of inflammatory cytokines was quantified by Real-Time PCR using the 2ΔΔct (75). Gene expression was normalized with actin and is represented as the fold increase to the untreated control. To confirm cytokine protein production and secretion from macrophages, media was collected and assayed by direct sandwich ELISA. IL-6, MIP-2, IL-1β, GM-CSF, TNF-α, and iNOS are primary mediators of the inflammatory response elicited by Gram-negative bacteria and were assayed to quantify the inflammatory responses elicited by *K. pneumoniae*.

Wild type outer membrane vesicles stimulate a stronger inflammatory response than the cellular outer membrane

To compare the inflammatory responses elicited by *K. pneumoniae* cellular OM and secreted OMVs, cytokine gene expression was quantified from macrophages treated with sequential dilutions of wild type OM and OMVs. All treatments resulted in significant up-regulation and secretion of these gene products. It has been shown that LPS stimulates IL-1β transcription without active secretion of the protein product (76). iNOS codes for inducible nitric

oxide synthase which produces nitric oxide used in macrophage oxidative burst. iNOS and IL-1b were only assayed by Real-Time PCR because neither of these inflammatory mediators produces a secreted, detectable protein product during the inflammatory response. As determined by both Real-Time PCR (Figure 3.1) and ELISA assay (Figure 3.2), OMVs stimulate a significantly more potent inflammatory response across all genes assayed than do purified OMs. The difference in these responses increased with decreasing bacterial dose, indicating the potency of these naturally secreted products. These significant differences were most apparent using the ELISA assay, indicating that post-transcriptional regulation of active secretion of these genes products may further amplify these responses. Transcriptional responses were most similar at the highest dose (0.01ug protein), which may indicate a saturation effect. Regardless, these doses still elicited significantly different levels of protein production. These data clearly demonstrate that the distinct composition of OMVs relative to the source membrane is recognized by macrophages to trigger a potent, distinctive inflammatory response.

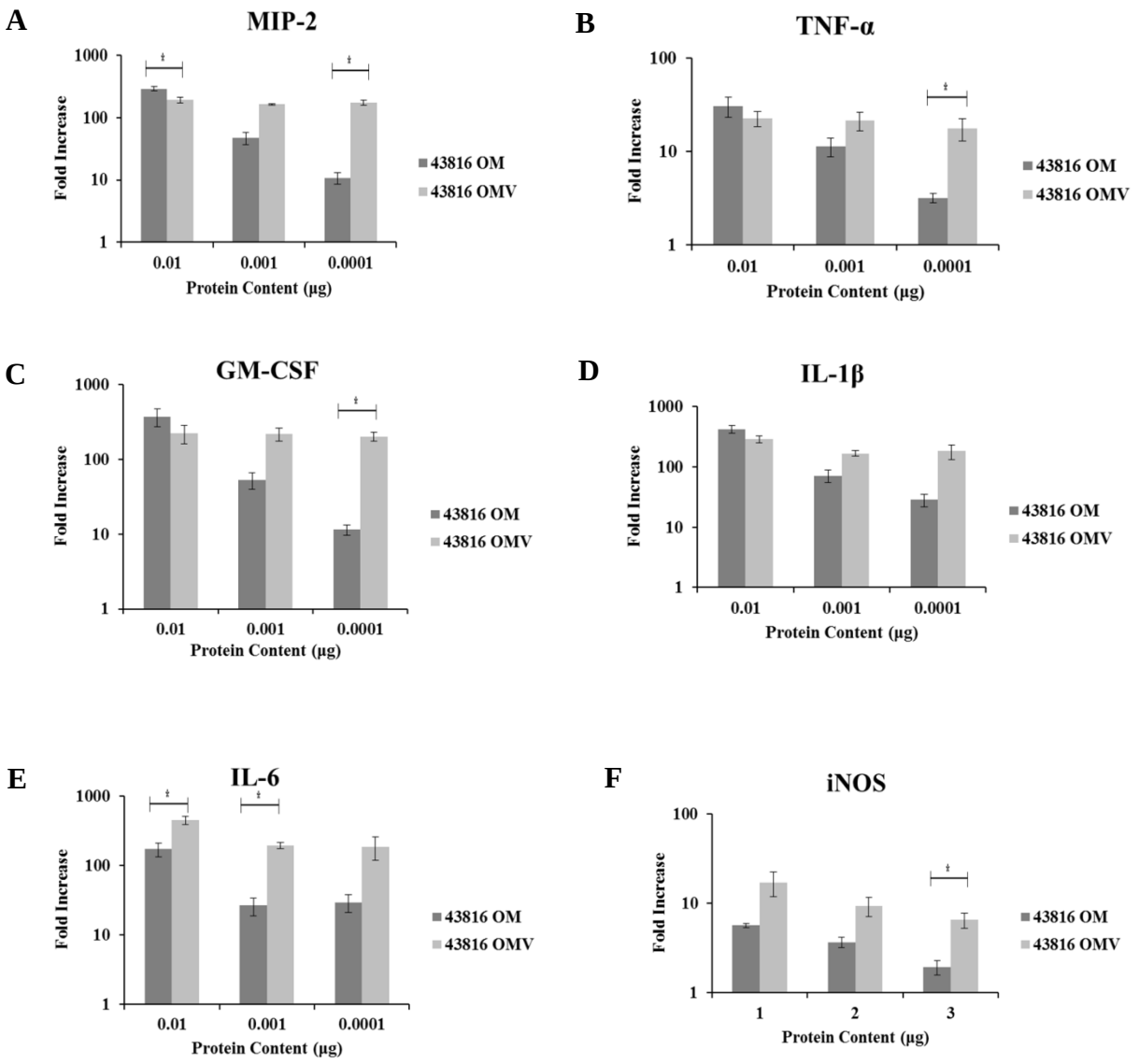

Figure 3.1 Wild type *K. pneumoniae* OMVs elicit a more potent inflammatory responses than the cellular OM. RAW 264.7 murine macrophages were treated with varying doses based on protein content of either the OM or OMV from *K. pneumoniae* 43816. Cells were incubated for 3 hrs and gene expression was quantified by real-time PCR for MIP-2 (A), TNF-α (B), GM-CSF (C), IL-1β (D), IL-6 (E), and iNOS (F). Error bars represent the SEM ($n \geq 4$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

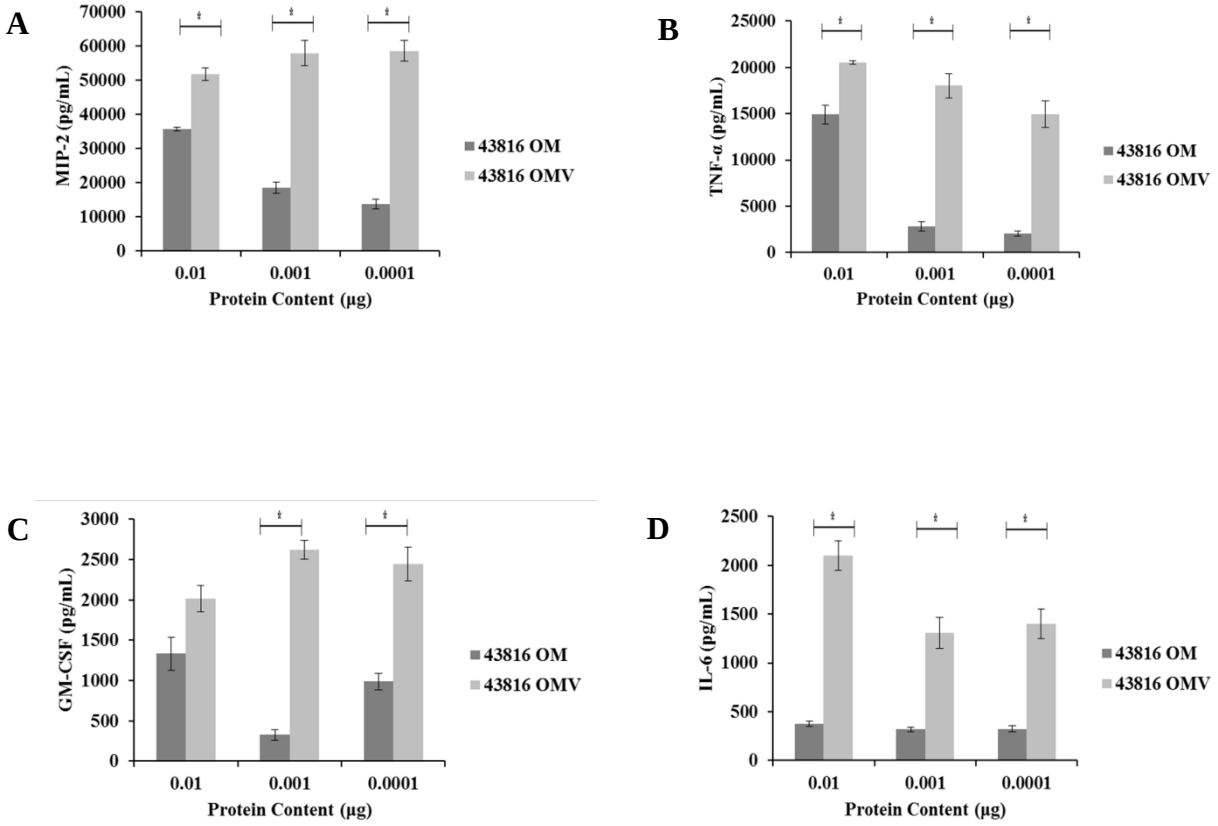

Figure 3.2 Wild type *K. pneumoniae* outer membrane vesicles stimulate a stronger responses than the cellular OM. RAW 264.7 murine macrophages were treated with increasing doses based on protein content of OM fractions or OMVs from *K. pneumoniae* 43816. Cells were incubated for 3 hrs and the culture media supernatants were collected and cytokine protein secretion was quantified by ELISA assays for MIP-2 (A), TNF-α (B), GM-CSF (C), and IL-6 (D). Error bars represent the SEM ($n \geq 5$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

O antigen-deficient outer membrane vesicles also stimulate a stronger inflammatory response than the cellular outer membrane

The previous proteomic study demonstrated that inactivation of the *wbbO* gene, responsible for the synthesis and attachment of the LPS O antigen, altered the protein profiles of the OM. Wild type OMVs elicited a stronger inflammatory response compared to the cellular OM. To confirm that this trend was also observed in the O antigen mutant, the inflammatory response generated from *K. pneumoniae ΔwbbO* OM and OMVs were also evaluated. As seen in Figure 3.3 and Figure 3.4, both inflammatory cytokine gene expression and protein secretion were significantly increased by treatment with the OMVs rather than the cellular OM. The lowest dose (0.0001 μg) displayed the greatest differences between the O antigen-deficient OM and OMV samples. At this dose cytokine gene expression was significantly higher due to treatment with *ΔwbbO* OMVs indicating that OMVs maintain their potency at lower doses (Figure 3.3). The differences between the potency of the OM and OMVs are further demonstrated by the amount of cytokine protein product secreted from macrophages. Compared to the cellular OM components derived from the *ΔwbbO* strain, cytokine protein production was greatly elevated by *ΔwbbO* OMV treatment for all proteins quantified (Figure 3.4). The results of this study confirm that distinct protein profiles of the OMVs results in a drastically increased inflammatory response compared to the cellular OM of the originating cell.

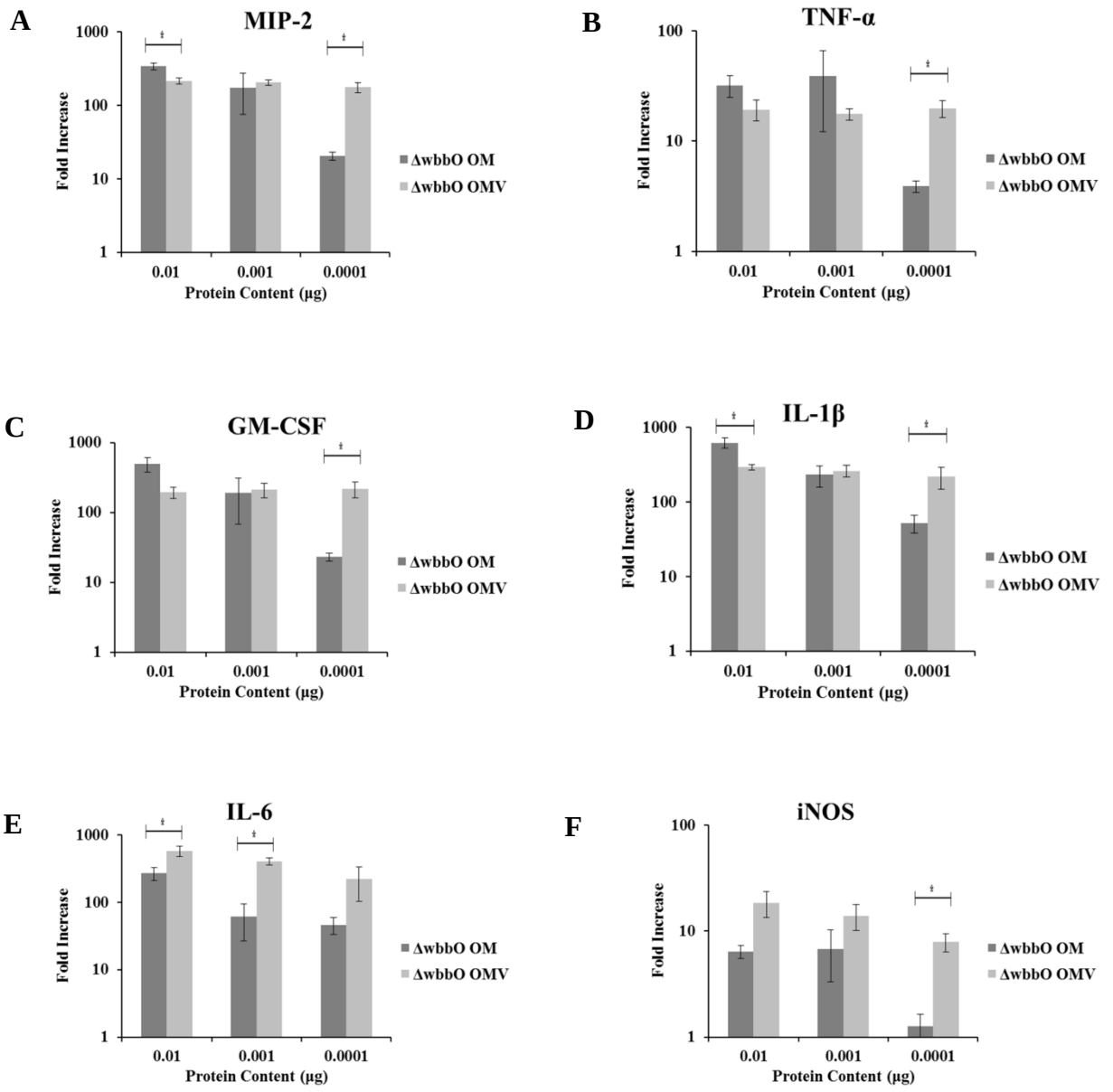

Figure3.3 O antigen-deficient *K. pneumoniae* OMVs elicits a more potent inflammatory responses than the cellular OM. RAW 264.7 murine macrophages were treated with varying doses of either the OM or OMV from *K. pneumoniae* Δ*wbbO.* Cells were incubated for 3 hrs and gene expression was quantified by real-time PCR for MIP-2 (A), TNF-α (B), GM-CSF (C), IL-1β (D), IL-6 (E), and iNOS (F). Error bars represent the SEM ($n \geq 4$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

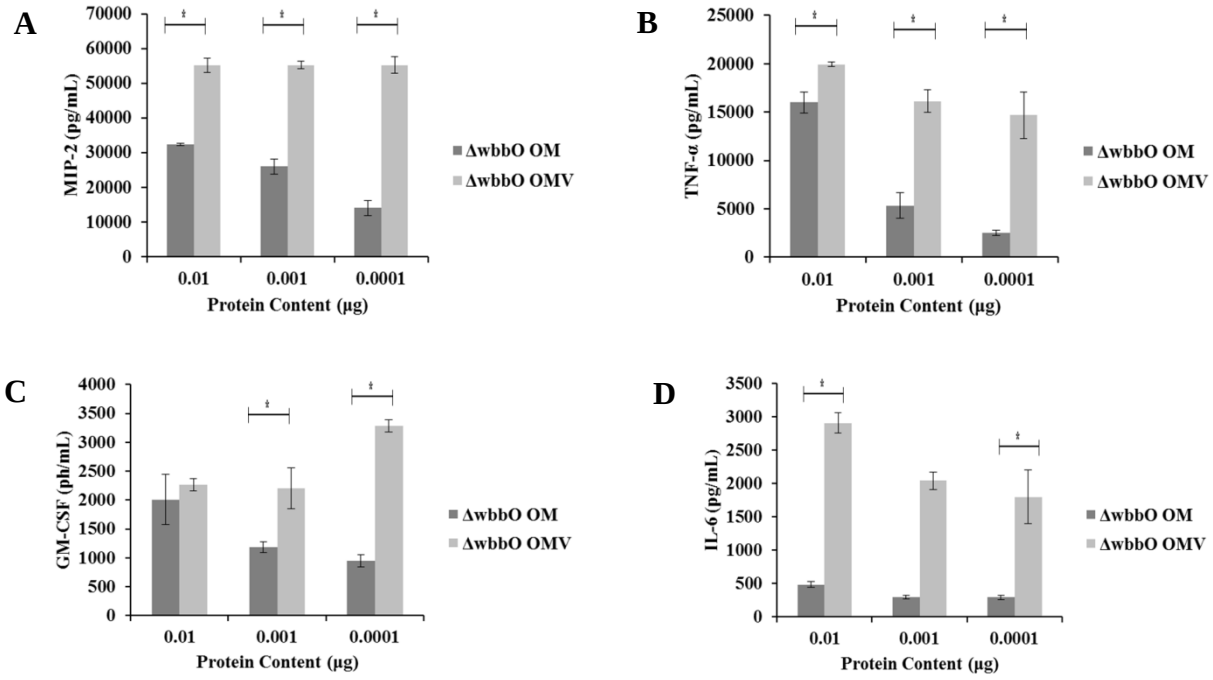

Figure 3.4 O antigen-deficient *K. pneumoniae* outer membrane vesicles stimulate a stronger responses than the cellular OM. RAW 264.7 murine macrophages were treated with different protein based doses of OM fractions or OMVs from *K. pneumoniae* Δ*wbbO*. Cells were incubated for 3 hrs and the culture media supernatants were collected and cytokine protein secretion was quantified by ELISA assays for MIP-2 (A), TNF-α (B), GM-CSF (C), and IL-6 (D). Error bars represent the SEM ($n \geq 5$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

The loss of the O antigen does not influence the inflammatory response elicited by the cellular outer membrane

We evaluated if changes to the OM resulting from O antigen loss would alter the inflammatory response. The current study demonstrated that the loss of the O antigen and resulting changes to the OM did slightly alter the inflammatory response elicited by the *K. pneumoniae* OM. At the highest dose inflammatory cytokine gene expression was relatively similar for both wild type and *ΔwbbO* OM treatments (Figure 3.5). However, at the lower doses the *ΔwbbO* OM stimulates a moderately, but not significantly, stronger response than the wild OM. As seen in Figure 3.6, there are some minor variations in cytokine protein secretion, but these variations are not significant between the wild type and O antigen-deficient OM responses. While the protein content of the OM was altered by the loss of the O antigen the inflammatory response was not drastically altered by these changes. These data indicate that the absence of the O antigen does not impact the inflammatory response elicited by the *K. pneumoniae* cellular OM components.

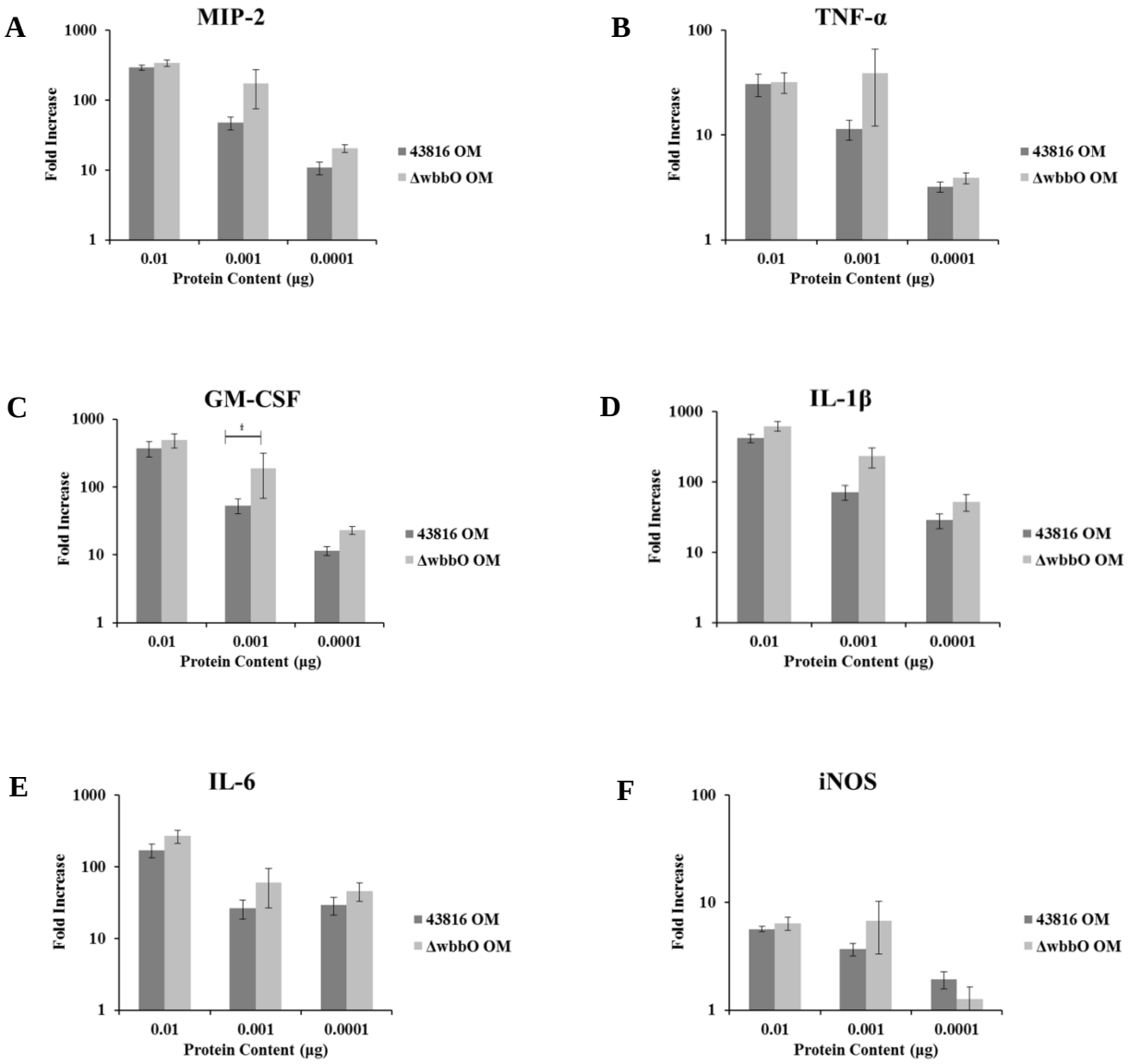

Figure 3.5 O antigen-deficient *K. pneumoniae* outer membrane fractions elicit more potent inflammatory responses than wild type OM fractions. RAW 264.7 murine macrophages were treated with increasing doses of OM fractions derived from *K. pneumoniae* 43816 or Δ*wbbO*. Cells were incubated for 3 hr and gene expression was quantified by real-time PCR for MIP-2 (A), TNF-α (B), GM-CSF (C), IL-1β (D), IL-6 (E), and iNOS (F). Error bars represent the SEM ($n \geq 4$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

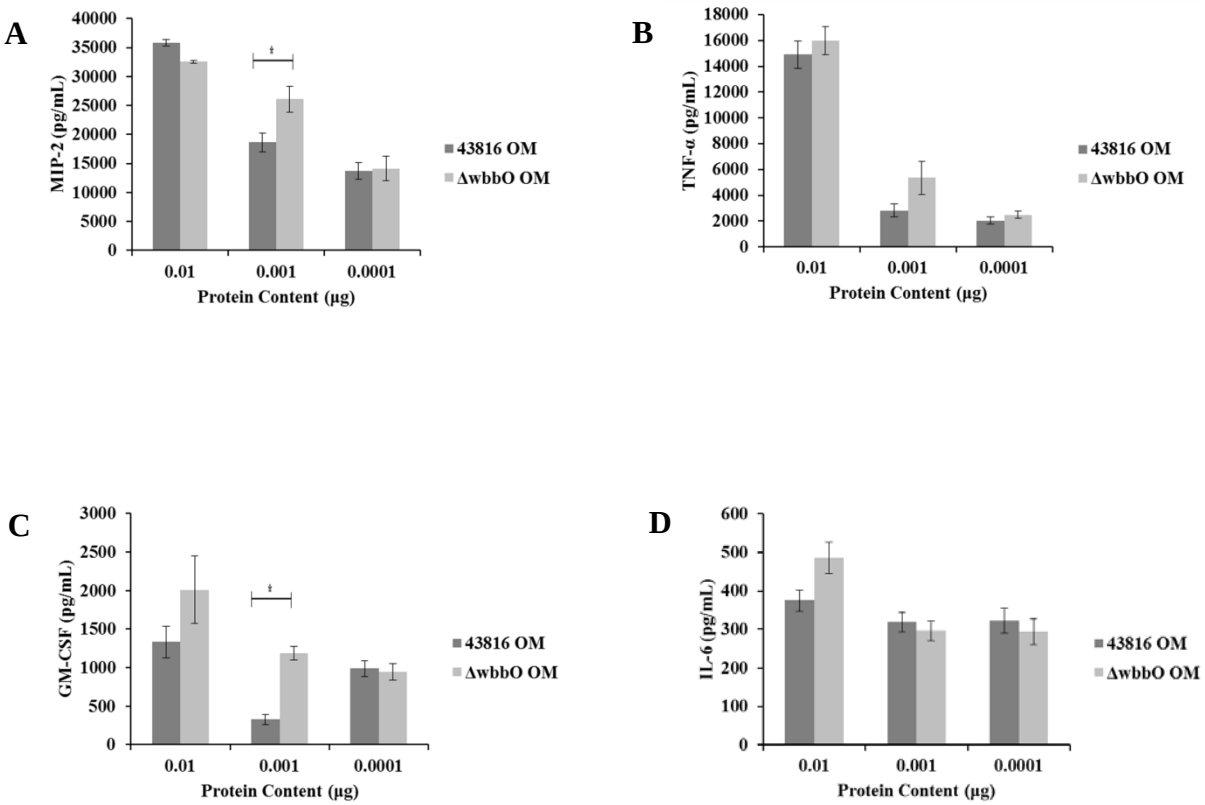

Figure 3.6 O antigen-deficient *K. pneumoniae* OM stimulates a stronger responses than the wild type OM. RAW 264.7 murine macrophages were treated with different doses of OM fractions from *K. pneumoniae* 43816 and Δ*wbbO.* Cells were incubated for 3 hrs and the culture media supernatants were collected and cytokine protein secretion was quantified by ELISA assays for MIP-2 (A), TNF-α (B), GM-CSF (C), and IL-6 (D). Error bars represent the SEM ($n \geq 5$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

O antigen-deficient OMVs elicit a potent IL-6 response

The previously described proteomic analysis revealed that O antigen-deficient OMVs were significantly less abundant in outer membrane proteins and were enriched in chaperones compared to wild type vesicles. Therefore it is likely that the changes of the OMV protein components associated with O antigen loss will alter the OMV mediated inflammatory response. For most of the cytokines assayed in this study, there were no significant differences in wild type and O antigen-deficient OMV responses (Figure 3.7). At the lower doses, IL-6, iNOS, and IL-1β gene expression was increased in macrophages treated with *ΔwbbO* OMVs. However, significance was only seen for IL-6. IL-6 protein secretion was significantly elevated in macrophages treated with *ΔwbbO* OMVs rather than wild type OMVs (Figure 3.8). There was also a significant increase in GM-CSF protein levels due to O antigen-deficient OMV exposure. This study indicates that the changes in OMV protein composition due to O antigen loss produce a distinct inflammatory response, one that triggers a potent IL-6 response in macrophages.

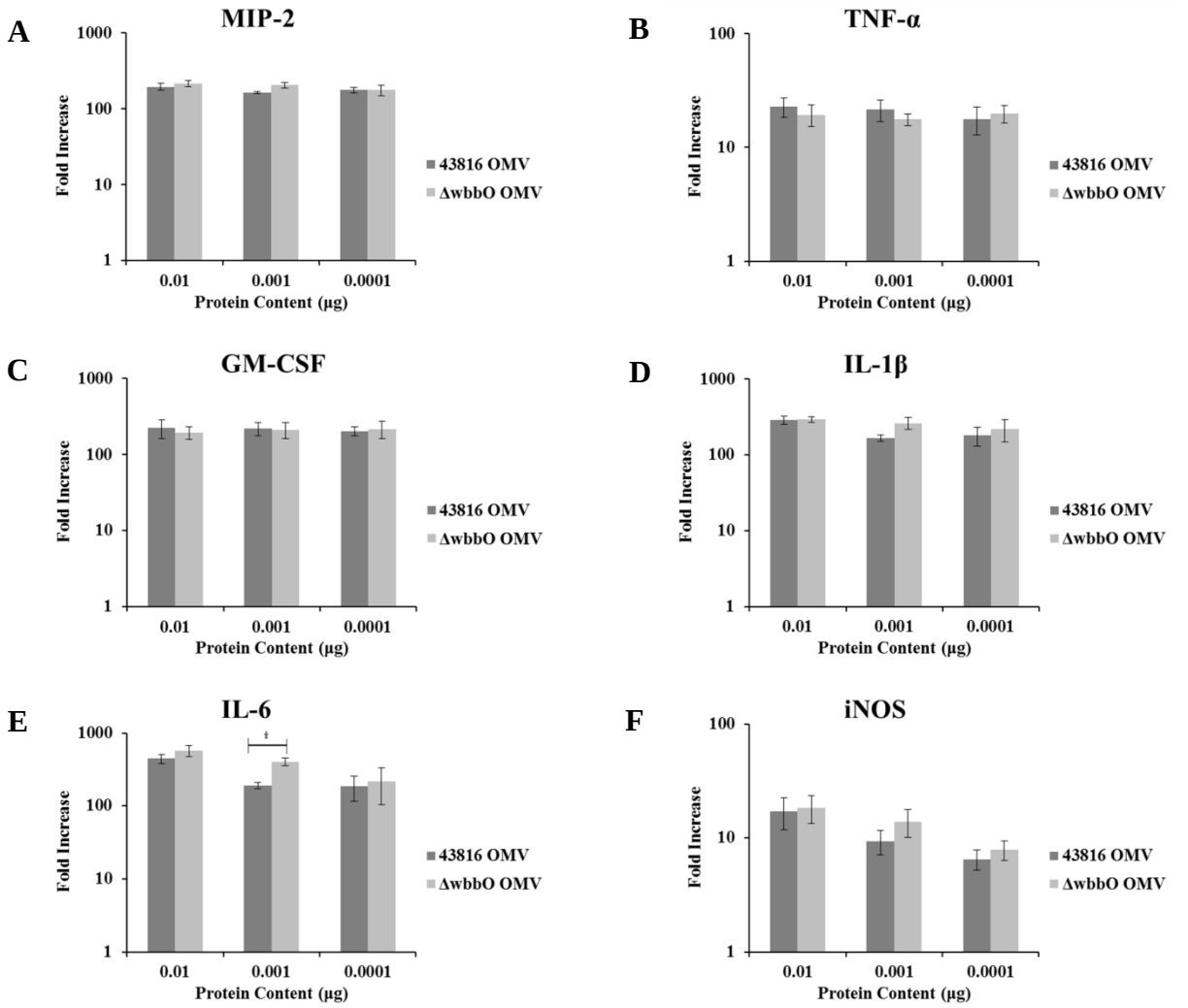

Figure 3.7 O antigen-deficient *K. pneumoniae* outer membrane OMVs elicit a more potent inflammatory responses than wild type OMVs. RAW 264.7 murine macrophages were treated with increasing doses of OMVs derived from *K. pneumoniae* 43816 or Δ*wbbO.* Cells were incubated for 3 hr and gene expression was quantified by real-time PCR for MIP-2 (A), TNF-α (B), GM-CSF (C), IL-1β (D), IL-6 (E), and iNOS (F). Error bars represent the SEM ($n \geq 4$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

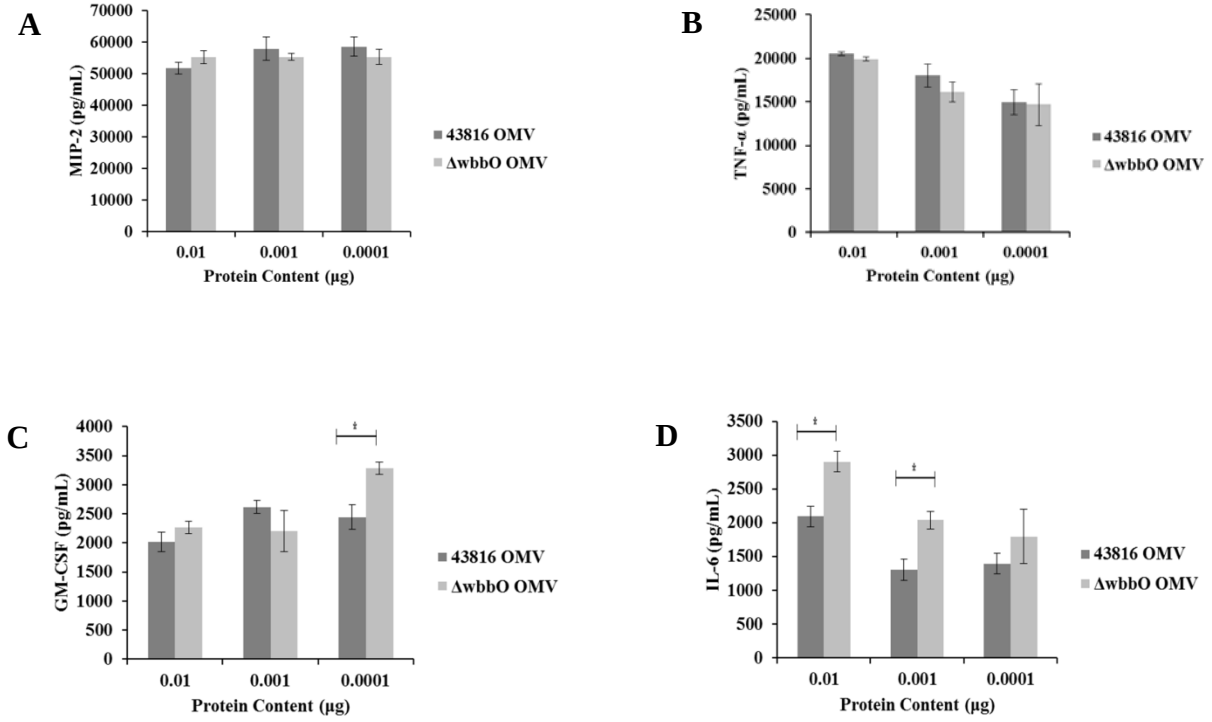

Figure 3.8 O antigen-deficient *K. pneumoniae* OM stimulates a stronger IL-6 responses than the wild type OM. RAW 264.7 murine macrophages were treated with different doses of OMVspurified from *K. pneumoniae* 43816 and Δ*wbbO*. Cells were incubated for 3 hrs and the culture media supernatants were collected and cytokine protein secretion was quantified by ELISA assays for MIP-2 (A), TNF-α (B), GM-CSF (C), and IL-6 (D). Error bars represent the SEM ($n \geq 5$). Statistical significance between groups is represented by horizontal brackets (* $P < 0.05$).

Discussion

The purpose of the present study was to do a comparative analysis of the inflammatory responses elicited by the outer membrane (OM) and secreted outer membrane vesicles (OMVs) derived from wild type and O antigen-deficient (Δ*wbbO*) *Klebsiella pneumoniae*.

The previous chapter demonstrated that the protein content of the OM and OMV were significantly different from one another. In the present study macrophages were exposed to OM fractions or purified OMVs derived from wild type and an O antigen mutant and the production of pro-inflammatory cytokines (IL-6, IL-1β, MIP-2, TNF-α, iNOS, and GM-CSF) were measured by Real-Time PCR and ELISA. When comparing the inflammatory responses stimulated by wild type OM components and the secreted OMVs, it was found that the OMVs rather than the cellular OM produced a significantly more potent inflammatory response. The previous proteomic study also concluded that the loss of the O antigen altered protein expression in both the OM and OMVs. Interestingly, the loss of the O antigen and resulting changes to the protein content of the membrane did not significantly impact the pattern of cytokine production in response to the OM but did alter the inflammatory responses elicited by the OMVs.

Because the protein content of OMVs is distinct from that of the cellular OM, it was anticipated that the inflammatory responses elicited by the OM and OMVs would also differ. Results from this study demonstrated that the OMVs produce a more powerful inflammatory response when compared to the OM. Even at the lowest dosage (0.0001 μg), both the transcript levels and protein products of inflammatory cytokine were significantly higher in response to OMV treatments. These data suggest that OMVs elicit a significantly stronger inflammatory response than the cellular OM.

This is the first study to directly compare the ability of the OM and OMV contents to stimulate the inflammatory response. Several *in vivo* studies support the conclusion that secreted OMVs rather than the cellular components stimulate a more potent immune response. Lee et al.(20120), reported that mice exposed to *K. pneumoniae* OMVs displayed more severe pathological changes in the lung than mice infected with live bacteria (30). Rat and mouse peritonitis models have analyzed the ability of OMVs to induce sepsis and also demonstrate the potent inflammatory response elicited by OMVs, most notably increased serum TNF-α and IL-6 levels. These studies demonstrated that OMVs from *Escherichia coli* induced sepsis in the absence of the originating bacterial cell (73, 74). These data demonstrate that OMVs themselves are highly potent virulence factors that elicit a stronger inflammatory response than the cells from which they are derived.

Both Lpp and OmpA are known to stimulate the production of inflammatory cytokines during *K. pneumoniae* infection and the enrichment of Lpp and OmpA likely contributes to the increased inflammatory response produced by *K. pneumoniae* OMVs (77, 78). The influence of outer membrane proteins should also be considered when evaluating these results since LPS and proteins are known to synergistically stimulate host immune responses. Results of the previous chapter revealed that compared to OMVs, the OM samples were significantly less enriched in many outer membrane proteins such as OmpA and Lpp. It has been shown the Lpp contributes to *K. pneumoniae in vivo* virulence by enhancing bacterial dissemination and stimulating a potent IL-6 response (77). Additionally, *K. pneumoniae* OmpA has been implicated in the adherence and inflammatory response resulting from *K. pneumoniae* derived pneumonia (78). As seen if Figures 3.1-3.4, OMVs elicit a drastically stronger inflammatory response than the OM. This

difference is likely due to OMVs being highly enriched in protein such as Lpp and OmpA that are known stimulators of the inflammatory response in Gram-negative bacteria.

The loss of the O antigen significantly altered the protein composition of *K. pneumoniae* OMVs. From the previous study, we concluded the Δ*wbbO* OMVs were less enriched in outer membrane porins and were more abundant in chaperones, compared to wild type OMVs. From the previous proteomic study it would be anticipated that a decrease in outer membrane porins, such as OmpA and OmpK35, would ultimately decrease the inflammatory response. However, studies in other bacterial species indicate that O antigen loss allows better access to the exposed LPS lipid A and protein PAMPs on the cell surface. This increased exposure to surface PAMPs provides better access to immune receptors. Based on this premise, the absence of the O antigen should ultimately result in a stronger inflammatory response (36, 79). Therefore we predicted that the loss of the LPS O antigen would result in O antigen-deficient *K. pneumoniae* producing an altered inflammatory cytokine response when compared to the wild type strain. This alteration could be due to either an increased inflammatory response due to better access to the surface because of loss of the O antigen, or decreased inflammation due to the altered protein content of these OMVs. Figures 3.5 and 3.6 demonstrate that O antigen loss only resulted in minor variations in inflammatory cytokine production patterns produced from the OM. The OM fractions derived from Δ*wbbO* produced a slightly enhanced cytokine response compared to wild type OM fractions. The relationship observed between wild type and O antigen-deficient OM cytokine responses was similar to that of wild type and O antigen-deficient OMVs. This result suggests the effects of O-antigen loss and altered membrane composition may have a balancing effect on the ultimate inflammatory response. The vesicles from the O antigen mutant produced a moderately stronger inflammatory response than wild type. With the exception of IL-6, the

differences in cytokine production observed between OMV treatments were not significantly different.

The role of the O antigen in *K. pneumoniae* immune response has been evaluated in other studies. A study by Shankar-Sinha et al. (2004), evaluated the effects of O antigen loss on *K. pneumoniae in vivo* pathogenicity. Mice intratracheally injected with live *K. pneumoniae* Δ*wbbO* bacteria had increased survival compared to mice injected with wild type cells (10). The O antigen mutant colonized lung tissue at the same rate as the wild type strain. However, the O antigen mutant failed to trigger bacteremia, which is identified by the presence of viable bacteria in the blood. If the localized inflammatory responses are potent, the tissue can be damaged, allowing for bacteria to enter the blood stream causing bacteremia. A failure to trigger bacteremia indicates that the O antigen mutant elicited a decreased inflammatory response. Our data differs from the *in vivo* study conducted by Shankar-Sinha et al. (2004) because our measured *in vitro* inflammatory response elicited by *K. pneumoniae* Δ*wbbO* was not attenuated, but rather indicates that the absence of the O antigen enhanced the inflammatory response *in vitro*. The findings of our study were supported by another *in vitro* study conducted by Evrard et al. (2010), in which whole UV killed *K. pneumoniae wecA* mutants deficient in O antigen were exposed to dendritic cells. In agreement with the findings of our study, Evrard et al. (2010), reported that the O antigen mutant generated increased TNF-α and IL-12 expression in dendritic cells (2). The results of the current study and those reported by Evrard et al. (2010), indicate that the absence of the O antigen enhances the inflammatory response *in vitro*; however, the study performed by Shankar-Sinha et al. (2004), suggests that the absence of the O antigen does not enhance the inflammatory response. There are several factors that may explain discrepencies seen between the *in vitro* and *in vivo* studies. First, the present study and the study conducted by

Evrard et al. (2010), respectively utilize OMVs and UV killed cells that are not actively replicating. It appears that the enhanced inflammatory response elicited by O antigen mutants in the *in vitro* studies may be associated with inactive bacterial components rather than live cells. Second, the *in vivo* study conducted by Shankar-Sinha et al. (2004) used live cells that were still capable of replicating and secreting cellular contents. This *in vivo* study exposed live bacterial cells to the complement system and multiple types of immune cells that negatively influence bacterial survival. Therefore, the findings reported by Shankar-Sinha et al. (2004), that the O antigen mutant failed to produce bacteremia may not be caused by a decreased inflammatory response, but could be due to the fact that the O antigen is required for resistance to complement mediated killing which results in decreased bacterial survival *in vivo* (5, 10).

Compared to wild type OMVs, Δ*wbbO* OMVs had a stronger inflammatory response and produced significantly higher IL-6 transcript and proteins levels. IL-6 is an inflammatory cytokine associated with Gram-negative bacterial infections. This cytokine is involved in the activation of the innate and adaptive immune responses by stimulating hepatocytes to produce acute-phase proteins involved in complement and by promoting the maturation of B cells. IL-6 activation is important for priming the immune response and increased serum IL-6 is often associated with strong inflammatory responses that induce fever, tissue damage at the site of infection, and the progression to sepsis (73, 80). Therefore, the association between the absence of the O antigen and increased IL-6 levels indicate that O antigen-deficient OMVs could greatly contribute to a potent inflammatory response elicited by *K. pneumoniae.* A study conducted by Odenbreit et. al. (2006), evaluated the role of the O antigen in the ability of *Helicobacter pylori* to induce IL-6 protein production. Results of that study indicate that the absence of the O antigen does not influence the ability of H. *pylori* cells to elicit an IL-6 response (69). This does not

agree with the findings of the present study. One explanation for this discrepancy is the fact that *H. pylori* has a Lipid A structure that greatly differs from the endotoxic Lipid A structure found in *K. pneumoniae* and other enteric bacteria. Both *E. coli* and *K. pneumoniae* have a Lipid A structure consisting of a bisphosphorylated sugar backbone containing hexa-acylated fatty acid chains that are 12-14 carbons long (6). Deviations from this structure, such as underacylation, longer acyl chains, and underphosphorylation tend to reduce LPS endotoxicity (6). *H. pylori* LPS is typically less endotoxic because it is monophosporylated, has longer acyl chains (14-16 carbons), and is underacylated (6). Compared to enteric LPS, *Limulus* amoebocyte lysate (LAL) assays revealed that *H. pylori* LPS has reduced endotoxicity by 100-500 fold. The absence of the O antigen may make the Lipid A portion more accessible for CD14 and TLR4, however, the *H. pylori* Lipid A structure tends to produce a much less potent inflammatory response than *K. pneumoniae* LPS. Other discrepancies between *K. pneumoniae* and *H. pylori* O antigen mutants' abilities to induce IL-6 is likely because the outer membrane content is different between the two species. Furthermore, the current study utilized *K. pneumoniae* OMVs rather than whole cells, as were used in the *H. pylori* study. Given that the protein content of the outer membrane tends to be different from the protein content of OMVs, it is difficult to directly compare the OMVs used in this study to the whole cells used by Odenbreit et al. (2006).

The absence of the O antigen may not be the causative factor resulting in IL-6 up-regulation and increased protein production. Several studies have demonstrated that GroEL (Hsp60), which was the most abundant protein identified in Δ*wbbO* OMVs, is present on the bacterial cell surface and stimulates the inflammatory response by interacting with LOX-1 on macrophages (40, 48). The presence of GroEL of the surface of *H. pylori* was also reported in the study performed by Odenbreit et al. (2006). In that study GroEL was associated with the post-

phagocytic IL-6 response elicited by *Helicobacter pylori* (69). The conclusion of the study conducted by Odenbreit et al. (2006), was that the presence of GroEL in the *H. pylori* OM was required for phagocytosis and IL-6 induction (69). Other chaperone proteins could possibly contribute to inflammation and virulence in *K. pneumoniae*, such as chaperones DegP, which was also highly enriched in Δ*wbbO* OMVs. It has been demonstrated that *K. pneumoniae* DegP mutants had higher LD_{50} doses in mouse models indicating that DegP contributes to *in vivo* virulence (81). Therefore, the absence of the O antigen may not directly influence the increased IL-6 response, but rather indirectly influence the inflammatory response by altering the protein content of OMVs.

This is the first study to directly compare the inflammatory response elicited by the cellular outer membrane components to those of the secreted OMVs. We have demonstrated that OMVs elicit a more potent inflammatory response than the purified cellular OM components. Moderate inflammatory responses are beneficial to the host by recruiting immune cells to the site of infection and priming the adaptive immune response (6). However, severe inflammatory responses can result in a systemic reaction that can lead to septic shock, organ failure, and death. The ability of OMVs to stimulate a potent inflammatory response indicates that OMVs play a significant role in *K. pneumoniae* pathogenicity. Shedding of LPS from *K. pneumoniae* has been shown to be required to induce cytotoxicity in airway epithelial cells (82). This shedding is most likely via OMV secretion. Most studies evaluating the role of the O antigen in immune responses use purified LPS, as opposed to LPS in its native, outer membrane integrated form (OMVs) to evaluate the role of the O antigen in activating the inflammatory response. Although absence of the O antigen from OMVs caused a slightly enhanced inflammatory response, these differences were not significant. The moderately stronger inflammatory response elicited by O antigen-

deficient OMVs cannot be directly attributed to the absence of the O antigen since other factors such as outer membrane proteins and chaperone enrichment were also altered in the O antigen mutant. Because there were alterations to both the proteins and LPS, we cannot solely confirm the role that the O antigen plays in the inflammatory response.

Chapter 4

Conclusion

This study was designed to evaluate the protein composition of the cellular outer membrane (OM) with that of the secreted outer membrane vesicles (OMVs) of *Klebsiella pneumoniae*. It was hypothesized that the protein composition of the secreted OMVs would be significantly different than the protein composition of the cellular OM. The inflammatory responses elicited by the OM and OMVs were also evaluated in this study. It was also anticipated that the OMVs would produce a distinct inflammatory response, most likely a stronger response, compared to the response of the OM. In the current study, the effects of the lipopolysaccharide (LPS) O antigen loss on protein composition and inflammatory responses of the OM and OMVs were also evaluated. Here it was predicted that the absence of the O antigen from the outer membrane would further alter the protein composition of both the cellular OM and the secreted OMVs. Furthermore, the changes to the membrane associated with O antigen loss would result in O antigen-deficient OM fractions and OMVs that would generate an altered inflammatory response, compared to the respective wild type OM and OMV responses.

Results of this study support the hypothesis that the OM and OMV sub-proteomes are significantly different. In this study, we found that there were many proteins that were either enriched in the OM or the OMVs, producing distinct OM and OMV protein profiles. This conclusion has been supported by several studies evaluating the protein profiles of the OM and OMVs by gel electrophoresis (25, 26). However, these studies only visualized the protein profiles by gel electrophoresis and did not have any OM proteomic-based data which to compare

the OMV protein content. In order to elucidate the membrane components involved in the selective sorting mechanism found in OMVs, more proteomics studies analyzing both the OM and OMV derived from multiple species of Gram-negative bacteria are needed.

Comparative proteomics-based OMV analyses are lacking in general, especially for *K. pneumoniae*. A study conducted by Lee et al. (2012), provided a small OMV protein dataset consisting of only 159 proteins and lacked functional explanations for these proteins with regards to *K. pneumoniae* survival and pathogenicity. With the exception of this limited proteomic study conducted by Lee et al. (2012), there is little information about the protein composition of *K. pneumoniae* OMVs Lee, et al. (30). Therefore more OM and OMV proteomic based studies using different *K. pneumoniae* strains are needed to fully understand the components and functionality of the membrane protein content.

Knowing that the OMVs have a dissimilar protein profile to the OM, it was anticipated that the OMVs would also produce an altered inflammatory response. Our study demonstrated that OMVs produce a much more potent inflammatory response than the cellular OM. The findings of our study have been supported by several studies reporting that OMVs stimulate a more potent inflammatory response than whole cells in *in vivo* (73, 74). The current study utilized purified OM fractions that form spheroplasts which structurally resemble vesicles. By using OM spheroplasts, we were able to directly compare the cellular OM and secreted OMV responses void of most of the intracellular components. However, this study did not identify the specific components responsible for the differences observed between the OM and OMV inflammatory responses. The next step for this study would be to evaluate the role of proteins that were highly enriched in OMVs in the inflammatory response. Comparing the OM and OMV inflammatory response elicited by knockout strains deficient in major outer membrane proteins,

such as OmpA or Lpp, would provide the detail needed to confirm which protein components are responsible for the potent OMV-mediated inflammatory response.

A secondary goal of this study was to evaluate the effect of O antigen loss on the protein composition of the OM and OMVs. It was predicted that the OM and OMVs derived from O antigen-deficient *K. pneumoniae* would have an altered protein profile, compared to the wild type strain. From our study we concluded that the absence of the O antigen slightly alters the protein content of the OM. However O antigen loss significantly altered the protein content of the OMVs. This trend has also been demonstrated in *P. gingivalis* and *P. aeruginosa*. These studies confirm our findings that while the OM protein content was not significantly changed by O antigen loss, the protein cargo of OMVs were significantly altered by the loss of the O antigen (25, 26). It is currently hypothesized that the selective sorting mechanism found in OMVs may be further altered by LPS modifications (23, 29). This hypothesis was formed based on data obtained analyzing bacterial species that contain negatively charged O antigen sugar residues. However, in *K. pneumoniae* LPS, the negative charge is imparted by galacturonic acid (GalU) residues on the core oligosaccharide (61). In order to confirm that the negative charge on the LPS influences protein integration into OMVs, the core oligosaccharide containing the GalU residues would need to be removed in *K. pneumoniae*. Repeating the proteomic study with a *K. pneumoniae WabH* mutant lacking charged GalU residues would provide the data needed to confirm that charged LPS influences protein packing into OMVs.

Given that O antigen-deficient OMVs having a significantly different protein profile than wild type OMVs, it was hypothesized that the inflammatory response would also be altered by the absence of the O antigen in OMVs. O antigen-deficient OMVs did have an altered inflammatory response compared to wild type OMVs. It is known that LPS and proteins simultaneously

stimulate the inflammatory response. Therefore, it is difficult to discern whether the loss of the O antigen directly influences inflammatory responses by providing TLRs with easier access to outer membrane PAMPs or indirectly influences the inflammatory response by altering the abundance of protein PAMPs. For instance, O antigen-deficient OMVs were highly enriched in GroEL and DegP compared to wild type OMVs. Other studies have reported that GroEL has been implicated in the activation of the inflammatory response (69). DegP has been associated with *K. pneumoniae* survival during infection (81). The enrichment of these proteins in O antigen-deficient OMVs could likely be responsible for the increased inflammatory response compared to wild type OMVs. Re-evaluating the inflammatory response after knocking out these proteins in the O antigen mutant would clarify if the proteins themselves or the absence of the O antigen causes O antigen-deficient OMVs to produce an increased inflammatory response.

In conclusion, this study was able to demonstrate that the OM and OMVs have significantly different protein profiles. This difference in protein profiles is likely responsible for the difference in the OM- and OMV-mediated inflammatory responses. The findings of this study also indicate that the loss of the O antigen significantly alters the protein content and inflammatory response elicited by OMVs. While this study provides a solid backbone for analyzing the protein content and inflammatory responses of the cellular OM and secreted OMVs in *K. pneumoniae*, future studies are needed to further elucidate the components of *K. pneumoniae* membrane and how they relate to the inflammatory response. Future studies could confirm the findings of this *in vitro* study by performing *in vivo* virulence studies using a mouse model. These future immunological studies should also compare the OM and OMVs to both live and UV killed whole bacterial cells to provide more insight into *K. pneumoniae* pathogenicity.

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Supplmental Table S1: The normalized spectral sums, cellular localization, and COG functional classification for the top 200 most abundant proteins across all samples identified in *Klebsiella pneumoniae* 43816 and Δ*wbb*O outer membrane (OM) and outer membrane vesicles (OMV). COG category descriptions are listed at the end of the file

Cellular Process & Signaling						Spectral Abundance			
Protein Description	Gene	Accession Number	Molecular Weight (kDa)	Subcellular localization	COG Category	ΔwbbO OM	ΔwbbO OMV	43816 OM	43816 OMV
Cell division protein FtsZ	ftsZ	R4Y5E6	40 kDa	Cyt	D	1.68	2.52	2.21	0.50
Chromosome partition protein MukB	mukB	R4YCA4	170 kDa	Cyt	D	0.79	0.00	2.85	0.50
Site-determining protein	minD	G0GIL6	30 kDa	Cyt	D	3.02	4.30	2.02	0.50
MglB protein	mglB	R4YDJ2	35 kDa	PP	G	4.78	0.56	5.08	0.00
OmpA protein ompA	ompA	R4YB96	41 kDa	OM	M	51.12	60.23	15.61	108.73
OmpK17 (OmpX)	ompk17	Q48427	18 kDa	OM	M	19.09	15.44	7.71	28.40
OmpC protein	ompC	R4Y807	41 kDa	OM	M	7.81	34.83	3.64	48.50
Outer membrane lipoprotein SlyB	slyB	G0GU11	15 kDa	OM	M	5.31	18.23	3.69	29.69
Tsx protein	tsx	R4YEE4	33 kDa	OM	M	3.89	13.61	3.71	23.73
TolC protein	tolC	R4YHT0	53 kDa	OM	M	7.35	3.08	5.04	10.89
Outer membrane protein assembly factor BamA	yaeT	R4Y5W4	90 kDa	OM	M	4.40	4.27	4.37	3.47
NlpD protein	nlpD	R4YHC8	40 kDa	OM	M	2.37	6.02	2.21	21.35
UTP--glucose-1-phosphate uridylyltransferase subunit GalU	galU	G0GHM2	33 kDa	Cyt	M	4.64	11.89	2.18	3.27
LPS-assembly protein LptD	lptD	R4YBZ4	85 kDa	OM	M	4.39	0.56	4.54	2.48
Scaffolding protein for murein-synthesising holoenzyme	mipA	G0GL03	28 kDa	OM	M	2.02	12.87	0.67	29.20
OmpW protein	ompW	R4YFW6	23 kDa	OM	M	4.21	3.02	3.02	1.29
Putative outer membrane lipoprotein	KPN2242_2257	G0GN82	22 kDa	OM	M	2.23	6.07	1.50	4.46
UDP-glucose pyrophosphorylase	galF	A2V7R3	33 kDa	Cyt	M	1.68	5.04	2.35	0.50
Penicillin-binding protein activator LpoA	lpoA	R4YEQ9	77 kDa	OM	M	0.87	3.61	0.34	2.28
OmpF protein	ompK35	O87753	40 kDa	OM	M	2.42	4.49	0.17	15.00
ABC transporter outer membrane lipoprotein	vacJ	G0GNY0	28 kDa	OM	M	0.87	3.05	1.18	5.36
OmpA protein ompA	ompA	R4YB97	41 kDa	OM	M	1.35	1.11	1.70	0.00
60 kDa chaperonin groEL	groEL	G0GGA7	57 kDa	Cyt	O	99.03	102.02	45.30	39.78
Chaperone protein DnaK	dnaK	R4Y5P6	68 kDa	Cyt	O	12.54	26.48	13.46	13.37
FKBP-type peptidyl-prolyl cis-trans isomeraseTrigger factor	tig	R4YHL8	48 kDa	Cyt	O	12.76	13.21	9.44	9.60
Heat shock protein 90	htpG	G0GQB6	71 kDa	Cyt	O	4.28	9.98	4.71	4.27
ClpB protein	clpB	R4Y6I5	96 kDa	Cyt	O	3.09	2.97	2.86	0.00
Alkyl hydroperoxide reductase subunit C	ahpC	G0GT45	21 kDa	Cyt	O	7.21	2.31	8.06	3.54
Lon protease	lon	R4YEB6	89 kDa	Cyt	O	1.24	2.63	3.21	0.00
DegP protein	degP	R4Y5J4	50 kDa	PP	O	1.68	7.96	2.50	5.75
alkyl hydroperoxide reductase subunit C	KPR_4367	R4YIQ4	22 kDa	Cyt	O	4.76	1.19	5.06	0.00
Co-chaperonin GroES	groES1	G0GGA6	10 kDa	Cyt	O	14.11	0.00	5.20	2.58
peptidyl-prolyl cis-trans isomerase	ybaU	R4YHL6	68 kDa	PP	O	1.18	0.56	2.18	1.49
FtsH protease regulator HflK	hflK	G0GGE4	46 kDa	IM	O	1.06	1.30	1.16	5.06
Peptidyl-prolyl cis-trans isomerase	KPR_0740	R4YBB0	23 kDa	PP	O	2.62	3.05	2.19	5.33
Stringent starvation protein A	sspA	G0GJQ5	24 kDa	Cyt	O	0.37	0.00	4.22	0.00
ATP-dependent protease ATPase subunit HslU	hslU	G0GRU2	50 kDa	Cyt	O	1.56	4.41	1.16	4.54
61 kDa chaperonin groEL	groEL	G0GGA8	57 kDa	Cyt	O	1.16	0.00	2.85	0.00
PrkA family serine protein kinase	KPR_2238	R4Y8E4	74 kDa	Cyt	T	1.48	0.56	2.20	0.00
Protein-export protein SecB	secB	G0GND1	17 kDa	Cyt	U	5.25	1.83	7.59	1.29
Protein translocase subunit SecA	secA	R4YC49	102 kDa	Cyt	U	1.77	0.00	1.83	0.00
AcrB protein	acrB	R4YE63	98 kDa	IM	V	1.68	1.11	2.03	0.99

Information Storage and Processing						Spectral Abundance			
Protein Description	Gene	Accession Number	Molecular Weight (kDa)	Subcellular localization	COG Category	ΔwbbO OM	ΔwbbO OMV	43816 OM	43816 OM
Elongation factor Tu ef-tu	ef-TU	G0GKM4	43 kDa	Cyt	J	51.11	66.86	37.38	75.81
Elongation factor G fusA	fusA	G0GKM5	78 kDa	Cyt	J	26.76	13.23	19.93	6.62
30S rib	rpsA	R4YGT0	61 kDa	Cyt	J	20.29	15.04	14.63	28.17
50S rib	rplB	G0GKL6	30 kDa	Cyt	J	5.83	23.22	3.52	12.41
30S rib	rpsD	G0GKJ6	24 kDa	Cyt	J	4.60	16.13	4.90	13.67
Elongation factor Ts	tsf	R4Y9M1	31 kDa	Cyt	J	10.88	1.19	8.40	0.00
30S rib	rpsC	G0GKL3	26 kDa	Cyt	J	7.04	18.09	3.71	10.09
50S rib	rplL	R4Y4G6	12 kDa	Cyt	J	7.92	8.09	8.74	8.91
Translation initiation factor IF-2	infB	G0GJK5	98 kDa	Cyt	J	7.68	4.16	5.05	0.00
50S rib	rplC	G0GKL9	22 kDa	Cyt	J	12.41	10.80	2.03	1.98
30S rib	rpsE	G0GKK2	18 kDa	Cyt	J	5.62	13.56	3.37	4.57
30S rib	rpsB	G0GLU3	27 kDa	Cyt	J	5.51	11.39	5.90	4.27
50S rib	rplF	G0GKK4	19 kDa	Cyt	J	5.41	6.63	4.19	8.11
50S ribosomal protein	rplQ	G0GKJ4	14 kDa	Cyt	J	1.12	8.12	0.67	5.56
50S ribosomal protein L15	rplO	G0GKK0	15 kDa	Cyt	J	5.92	7.70	3.00	12.38
Polyribonucleotide nucleotidyltransferase	pnp	R4YIY4	76 kDa	Cyt	J	6.08	3.34	4.88	0.00
30S ribosomal protein S9	rpsI	G0GJQ6	15 kDa	Cyt	J	4.26	4.88	2.52	5.56
50S ribosomal protein L14	rplN	G0GKK9	14 kDa	Cyt	J	3.23	4.49	3.88	5.56
Glycine--tRNA ligase beta subunit	glyS	R4YFI7	76 kDa	Cyt	J	2.60	2.78	4.50	0.00
50S ribosomal protein L21	rplU	G0GJM3	12 kDa	Cyt	J	8.77	5.71	2.50	9.87
30S ribosomal protein S10	rpsJ	G0GKM0	12 kDa	Cyt	J	2.47	9.53	2.35	7.02
Aspartate--tRNA ligase	aspS	G0GJG6	66 kDa	Cyt	J	3.64	1.19	5.02	0.00
50S ribosomal protein L13	rplM	R4YIZ5	19 kDa	Cyt	J	2.99	3.05	3.19	2.78
Alanine--tRNA ligase	alaS	R4Y667	96 kDa	Cyt	J	3.97	0.00	3.53	0.00
50S ribosomal protein L18	rplR	G0GKK3	13 kDa	Cyt	J	3.04	9.28	0.51	12.68
Leucine--tRNA ligase	leuS	R4YH52	97 kDa	Cyt	J	2.10	0.56	3.53	0.00
Lysine--tRNA ligase	lysS	G0GV85	58 kDa	Cyt	J	1.72	2.41	3.72	0.00
50S ribosomal protein L22	rplV	G0GKL4	12 kDa	Cyt	J	3.16	5.68	2.00	16.08
50S ribosomal protein L4	rplD	R4Y5C4	22 kDa	Cyt	J	4.10	6.18	2.52	0.99
50S ribosomal protein L9	rplI	G0GH43	16 kDa	Cyt	J	6.16	3.74	2.87	5.33
Isoleucine--tRNA ligase	ileS	R4Y983	103 kDa	Cyt	J	2.77	0.00	3.86	0.00
50S ribosomal protein L10	rplJ	G0GSV3	18 kDa	Cyt	J	4.18	7.54	1.70	0.00
Ribonuclease E	rne	R4Y882	119 kDa	Cyt	J	4.76	1.11	3.66	0.00
50S ribosomal protein L5	rplE	G0GKK7	20 kDa	Cyt	J	3.62	4.83	2.89	2.28
30S ribosomal protein S7	rpsG	G0GKM6	18 kDa	Cyt	J	2.10	8.04	2.34	4.57
50S ribosomal L23	rplW	G0GKL7	11 kDa	Cyt	J	1.81	3.79	0.51	7.61
30S ribosomal protein S6	rpsF	R4YAT3	15 kDa	Cyt	J	3.81	4.83	1.36	0.00
Valine--tRNA ligase	valS	R4YAZ9	108 kDa	Cyt	J	0.19	0.00	1.84	0.00
30S ribosomal protein S13	rpsM	G0GKJ8	13 kDa	Cyt	J	1.54	3.02	2.36	0.00
Glutathione synthetase	gshB	G0GGZ6	35 kDa	Cyt	J	3.32	0.00	3.03	0.00
Elongation factor Tu ef-tu	ef-TU	G0GKM5	43 kDa	Cyt	J	4.20	0.66	1.68	0.99
DNA-directed RNA polymerase	rpoC	G0GSV6	155 kDa	Cyt	K	10.51	14.67	11.08	3.27
DNA-directed RNA polymerase subunit beta	rpoB	R4Y490	152 kDa	Cyt	K	9.45	12.57	12.56	4.76
DNA-directed RNA polymerase subunit alpha	rpoA	G0GKJ5	36 kDa	Cyt	K	6.05	10.14	6.08	9.40
PutA protein	putA	R4YBC6	145 kDa	Cyt	K	2.28	0.64	5.17	0.00
Transcription termination factor Rho	rho	R4Y7C7	50 kDa	Cyt	K	3.97	1.88	3.21	4.84
Transcription termination/antitermination protein NusA	nusA	G0GJK6	55 kDa	Cyt	K	4.09	1.22	3.19	0.50
Recombinase A	recA	G0GRR9	38 kDa	Cyt	L	0.56	11.67	0.66	2.78
DNA gyrase subunit B	gyrB	R4Y6T5	90 kDa	Cyt	L	0.37	0.64	1.84	0.00
HrpA protein	hrpA	R4Y9R0	149 kDa	Cyt	L	0.37	0.66	0.33	3.54
DNA gyrase subunit A	gyrA	R4Y7H5	97 kDa	Cyt	L	1.35	0.56	2.01	0.00

Metabolism						Spectral Abundance			
Protein Description	Gene	Accession Number	Molecular Weight (kDa)	Subcellular localization	COG Category	ΔwbbO OM	ΔwbbO OMV	43816 OM	43816 OM
PflB protein formate acetyltransferase pflB	pflB	R4YCC3	85 kDa	Cyt	C	12.24	13.58	19.35	4.04
ATP synthase subunit beta atpB	atpB	G0GQ84	50 kDa	IM	C	15.52	22.18	16.67	7.81
AceF protein	aceF	R4Y5U7	66 kDa	Cyt	C	10.06	33.73	5.65	43.70
ATP synthase subunit alpha	atpA	G0GQ86	55 kDa	IM	C	12.31	23.45	13.62	11.09
Aldehyde-alcohol dehydrogenase	adhE	R4YFG8	96 kDa	Cyt	C	12.13	9.74	16.46	0.99
Pyruvate dehydrogenase E1 component	aceE	R4Y5F9	99 kDa	Cyt	C	12.30	4.99	13.92	0.99
Dihydrolipoyl dehydrogenase	lpdA	R4Y9G6	51 kDa	Cyt	C	6.51	23.32	5.70	9.03
NarG protein	narG	R4YB12	141 kDa	IM	C	2.71	5.52	5.70	10.32
Succinyl-CoA ligase [ADP-forming] subunit beta	sucC	R4YCF4	42 kDa	Cyt	C	7.22	8.57	7.09	4.27
Dihydrolipoamide succinyltransferase	sucB	G0GTX2	44 kDa	Cyt	C	3.81	9.76	7.07	2.28
Aconitate hydratase 2	acnB	R4Y5U9	94 kDa	Cyt	C	4.69	3.42	6.88	0.99
Isocitrate dehydrogenase [NADP]	icdA	F1DFL4	46 kDa	Cyt	C	7.64	1.11	6.05	1.29
SucA protein	sucA	R4YH31	105 kDa	Cyt	C	6.62	4.05	5.84	0.00
Succinate dehydrogenase, flavoprotein subunit	sdhA	R4YD02	63 kDa	IM	C	3.87	13.05	4.04	3.97
Malate dehydrogenase	mdh	R4YIZ6	32 kDa	Cyt	C	8.90	0.56	5.94	0.00
Succinyl-CoA ligase [ADP-forming] subunit alpha	sucD	G0GUL4	30 kDa	Cyt	C	3.76	1.67	4.18	11.46
Aldehyde dehydrogenase (NAD) family protein	KPR_1299	R4YA65	55 kDa	Cyt	C	6.56	0.00	6.14	0.00
Glycerol kinase	glpK	R4Y4P0	56 kDa	Cyt	C	2.53	2.39	5.08	1.49
ATP synthase subunit b	atpF	G0GQX5	17 kDa	IM	C	4.35	6.24	2.16	5.45
ATP synthase gamma chain	atpG	G0GQ85	32 kDa	Cyt	C	1.43	0.00	3.90	0.00
Citrate synthase	cisY	R4YH33	48 kDa	Cyt	C	3.43	0.00	4.69	0.00
CydA protein	cydA	R4YI62	58 kDa	IM	C	5.18	3.61	3.04	1.49
Phosphoenolpyruvate carboxykinase	pckA	R4YFN7	58 kDa	Cyt	C	2.31	1.11	3.37	0.00
fumarate reductase flavoprotein subunit	frdA	R4Y4K6	65 kDa	Cyt	C	1.87	1.22	3.21	0.50
Inorganic pyrophosphatase	ppa	G0GH67	20 kDa	Cyt	C	5.59	3.61	1.69	0.00
MmsA protein	mmsA	R4Y4S8	54 kDa	Cyt	C	1.62	0.00	3.01	0.00
NarH protein	narH	R4YAC3	58 kDa	IM	C	1.16	0.66	2.52	5.06
NuoG protein	nuoG	R4Y7F5	100 kDa	Cyt	C	1.39	0.56	2.33	0.00
NADH-quinone oxidoreductase subunit C/D	nuoCD	G0GN40	69 kDa	IM	C	1.06	0.66	3.20	0.99
PutA protein	putA	R4YBC6	145 kDa	Cyt	E	2.28	0.64	5.17	0.00
Urocanate hydratase	hutU	R4YCT2	62 kDa	Cyt	E	7.55	2.47	8.09	0.00
Histidine ammonia-lyase	hutH	R4YI45	53 kDa	Cyt	E	7.37	1.11	7.73	0.00
putative lysine decarboxylase	KPR_4061	R4YHB3	82 kDa	Cyt	E	4.34	0.00	6.01	0.00
Aspartate ammonia-lyase	aspA	G0GGA2	52 kDa	Cyt	E	3.87	0.56	5.38	0.00
Serine hydroxymethyltransferase	glyA	R4Y647	45 kDa	Cyt	E	1.62	1.22	3.70	0.99
OppA protein	oppA	R4YAA5	61 kDa	PP	E	1.91	0.00	5.22	0.00
Glycine dehydrogenase [decarboxylating]	gcvP	R4YEZ3	101 kDa	Cyt	E	0.93	0.00	4.00	0.00
PepD protein	pepD	R4Y600	52 kDa	Cyt	E	0.87	0.00	3.36	0.50
D-amino acid dehydrogenase small subunit	dadA	R4YB55	47 kDa	IM	E	1.24	1.30	2.03	1.79
30S ribosomal protein S11	rpsK	G0GKJ7	14 kDa	Cyt	E	2.52	3.71	1.18	5.86
Cysteine synthase	cysK	Q0ZB89	35 kDa	Cyt	E	2.29	0.56	2.53	0.00
AspC protein	aspC	R4YBN3	43 kDa	Cyt	E	2.31	1.67	3.73	0.00
Ribose-phosphate pyrophosphokinase	prsA	R4YFK8	34 kDa	Cyt	E/F	2.08	3.61	2.52	0.50
Purine nucleoside phosphorylase DeoD-type	deoD	R4Y5N1	26 kDa	Cyt	F	4.55	1.22	3.39	0.00
Inosine-5'-monophosphate dehydrogenase	guaB	G0GPX1	52 kDa	Cyt	F	3.45	0.56	4.87	0.50
Ribonucleoside-diphosphate reductase	nrdA	R4YF73	86 kDa	Cyt	F	2.35	2.52	2.36	0.50
Adenylosuccinate synthetase	purA	G0GGE8	47 kDa	Cyt	F	1.87	1.11	3.05	0.00
Uridylate kinase	pyrH	G0GMI7	26 kDa	Cyt	F	0.81	0.00	1.52	0.00
CTP synthase	pyrG	G0GTD7	60 kDa	Cyt	F	1.24	1.30	2.33	0.00
Glyceraldehyde-3-phosphate dehydrogenase A	gapA	G0GL06	35 kDa	Cyt	G	53.91	31.35	19.88	24.13
Enolase	enoj	R4YIH7	46 kDa	Cyt	G	17.52	22.36	15.21	7.81

Phosphoglycerate kinase	pgk	G0GGY4	41 kDa	Cyt	G	9.60	5.72	16.09	0.00
FbaA protein	fbaA	R4YF09	37 kDa	Cyt	G	15.27	3.53	11.27	0.00
Phosphoenolpyruvate synthase	ppsA	R4Y9N4	87 kDa	Cyt	G	5.05	4.85	6.72	1.29
Pyruvate kinase	pykF	R4Y9K5	51 kDa	Cyt	G	5.71	1.67	7.94	1.29
Transketolase	tktA	R4Y8C3	72 kDa	Cyt	G	4.89	3.61	7.39	1.29
Aconitase	acnA	R4Y890	98 kDa	Cyt	G	4.69	1.30	5.90	1.29
6-phosphogluconate dehydrogenase, decarboxylating	gnd	G0LD05	51 kDa	Cyt	G	4.85	3.50	6.86	0.00
Transaldolase	talB	R4Y5A8	35 kDa	Cyt	G	4.53	0.56	6.39	0.00
Pyruvate kinase	pykA	R4YAU5	51 kDa	Cyt	G	3.93	5.38	3.90	1.79
Phosphoenolpyruvate-protein phosphotransferase	ptsI	R4Y7Q8	63 kDa	Cyt	G	4.18	2.52	5.06	4.54
Phosphoglyceromutase	gpmA	G0GUN2	28 kDa	Cyt	G	8.78	1.75	4.73	2.58
Glucose-6-phosphate isomerase	pgi	R4Y4E6	61 kDa	Cyt	G	2.86	0.64	3.69	0.00
FbaB protein	fbaB	R4YDN3	38 kDa	Cyt	G	4.66	1.19	4.56	0.00
Triosephosphate isomerase	tpiA	G0GP66	27 kDa	Cyt	G	4.90	0.00	3.40	3.54
ScrY protein	scrY	R4YDP8	56 kDa	OM	G	1.71	2.52	1.69	6.25
Pgm protein	pgm	R4YD15	57 kDa	Cyt	G	3.49	0.56	3.85	0.00
Putative myo-inositol catabolism protein	KPN2242_0146	G0GI16	71 kDa	Cyt	G	1.43	0.56	4.07	0.00
Klebsiella pneumoniae subsp. rhin	KPR_2139	R4Y7M6	51 kDa	IM	G	0.87	4.27	3.16	6.32
Crr protein	crr	R4YEV8	18 kDa	Cyt	G	2.91	1.86	2.88	1.79
Phosphopentomutase	deoB	G0GJW3	44 kDa	Cyt	G	2.47	0.00	3.73	0.00
MalE protein	malE	R4Y4F1	41 kDa	PP	G	1.31	0.00	2.69	0.00
2,3-bisphosphoglycerate-independent phosphoglycerate mutase	gpmI	R4YFY9	56 kDa	Cyt	G	1.54	0.00	2.56	0.00
6-phosphofructokinase	pfkA	R4Y9P2	35 kDa	Cyt	G	1.56	0.00	2.37	0.00
Glyceraldehyde-3-phosphate dehydrogenase A	gapA	G0GL07	35 kDa	Cyt	G	3.29	1.11	2.52	0.00
Glutathione synthetase	gshB	G0GGZ6	35 kDa	Cyt	H	3.32	0.00	3.03	0.00
Vitamin B12 transporter BtuB	btuB	R4Y432	68 kDa	OM	H	0.00	3.61	0.67	13.34
S-adenosylmethionine synthase	metK	R4YFG0	42 kDa	Cyt	H	2.95	0.56	2.70	0.00
HemX protein	hemX	R4Y7E1	43 kDa	OM	H	1.86	3.55	1.69	0.99
Carbamoyl-phosphate synthase large chain	carB	R4Y993	118 kDa	Cyt	I	1.54	1.83	2.65	0.00
FabB protein	fabB	R4Y7C9	38 kDa	Cyt	I	2.10	6.34	2.03	5.33
Acetyl-coenzyme A synthetase	acs	G0GUJ0	72 kDa	Cyt	I	2.24	0.56	3.87	0.00
PflB protein formate acetyltransferase pflB	pflB	R4YCC4	85 kDa	Cyt	C	2.89	-0.24	2.70	0.38
PflB protein formate acetyltransferase pflB	pflB	R4YCC4	85 kDa	Cyt	C	2.83	-0.35	2.64	0.32
ATP synthase subunit beta atpB	atpB	G0GQ85	50 kDa	IM	C	2.78	-0.45	2.57	0.27
AceF protein	aceF	R4Y5U8	66 kDa	Cyt	C	2.73	-0.56	2.51	0.21
ATP synthase subunit alpha	atpA	G0GQ87	55 kDa	IM	C	2.68	-0.66	2.45	0.16
Aldehyde-alcohol dehydrogenase	adhE	R4YFG9	96 kDa	Cyt	C	2.63	-0.77	2.38	0.10
Pyruvate dehydrogenase E1 component	aceE	R4Y5F10	99 kDa	Cyt	C	2.57	-0.87	2.32	0.04

Metabolism						Spectral Abundance			
Protein Description	Gene	Accession Number	Molecular Weight (kDa)	Subcellular localization	COG Category	ΔwbbO OM	ΔwbbO OMV	43816 OM	43816 OM
Murein lipoprotein KPN2242_	KPN2242_1363	G0GGR8	8 kDa	OM	*	12.51	57.83	5.89	122.11
Klebsiella pneumoniae subsp. rhin	KPR_1266	R4Y671	12 kDa	PP	*	2.00	15.17	1.68	13.26
Murein lipoprotein KPN2242_	KPN2242_1363	G0GGR9	8 kDa	OM	*	0.19	0.66	0.00	0.00
ABC transporter substrate binding protein	KPR_2917	R4YH39	70 kDa	Cyt	R	11.63	0.66	8.31	0.00
Outer membrane lipoprotein	yraP	G0GIV4	20 kDa	OM	R	1.92	19.02	1.69	24.81
ATP-binding cassette domain of elongation factor 3	KPR_0924	R4Y955	62 kDa	Cyt	R	4.54	3.19	4.03	0.00
Global DNA-binding transcriptional dual regulator H-NS	hns	G0GHM1	15 kDa	Cyt	R	3.76	4.83	4.21	0.50
NAD(P)H dehydrogenase (quinone)	wrbA	R4YC37	21 kDa	Cyt	R	3.40	0.00	6.25	0.00
Uncharacterized protein	yqjD	G0GIT3	11 kDa	Unknown	S	4.26	8.04	3.36	2.28
YfaZ protein	yfaZ	R4Y5J6	19 kDa	OM	S	0.81	7.88	0.84	20.79
Outer membrane protein assembly factor BamB	yfgL	G0GPX6	42 kDa	OM	S	2.52	3.71	2.18	4.96
Outer membrane protein assembly factor BamC	bamC	R4Y768	37 kDa	OM	S	1.74	0.56	3.70	1.49
YbaY protein	ybaY	R4YCW8	15 kDa	OM	S	1.68	2.47	2.36	3.27

COG Category	
	Cellular Process
D	Cell cycle control, cell division, chromosome partitioning
M	Cell wall/membrane/envelope biogenesis
O	Post-translation modification, protein turnover, chaperones
T	Signal Transduction mechanisms
U	Intracellular trafficking, secretion, and vesicular transport
V	Defense mechanism
	Information
J	Translation, ribosomal structure and biogenesis
K	Transcription
	Metabolism
L	Replication, Recombination and repair
C	Energy production and conversion
E	Amino acid transport and metabolism
F	Nucleotide transport and metabolism
G	Carbohydrate metabolism and transport
H	Coenzyme transport and metabolism
I	Lipid transport and metabolism
	Poorly
P	Inorganic ion transport and metabolism
*	Not in COG genes
R	General function prediction only

S	Function unknown

Supplemtal Table S2: P-values were assessed by single factor ANOVA to determine statistical significance for enrichment of outer membrane proteins and and chaperones between *Klebsiella pneumoniae* Δ*wbb*O and 43816 outer membrane (OM) and outer membrane vesicle (OMV) samples.

					COG Category		OM-OMV			
Protein Description	**Gene**	**Accession #**	**Molecular Weight**	**Localization**	**Cellular processes**	**Metabolism**	**Poorly Characterized**	**Δ*wbb* O**	**43816**	**OM-OM**	**OMV-OMV**
OmpA protein ompA	ompA	R4YB96	41 kDa	OM	M			0.76043643	0.001567519	0.239160262	0.040799283
Murein lipoprotein KPN2242_	KPN2242_1363	G0GGR8	8 kDa	OM			*	0.059886196	0.003423883	0.112310133	0.064297019
OmpK17 (OmpX)	ompk17	Q48427	18 kDa	OM	M			0.751602804	0.002886961	0.336827721	0.035396224
OmpC protein	ompC	R4Y807	41 kDa	OM	M			0.166428173	0.075595115	0.279751268	0.606100641
Outer membrane lipoprotein SlyB	slyB	G0GU11	15 kDa	OM	M			0.023756437	0.002450687	0.163487071	0.091972113
Tsx protein	tsx	R4YEE4	33 kDa	OM	M			0.006090545	0.013556267	0.770990195	0.116109575
TolC protein	tolC	R4YHT0	53 kDa	OM	M			0.076491238	0.003378913	0.290881701	0.000403276
Outer membrane lipoprotein	yraP	G0GIV4	20 kDa	OM			R	0.033299327	0.136541449	0.689569758	0.691006843
Outer membrane protein assembly factor BamA	yaeT	R4Y5W4	90 kDa	OM	M			0.932369383	0.809913978	0.982941434	0.830103581
NlpD protein	nlpD	R4YHC8	40 kDa	OM	M			0.103661498	0.096204025	0.917190282	0.159794846
YfaZ protein	yfaZ	R4Y5J6	19 kDa	OM			S	0.005521834	0.000244548	0.963636031	0.002765098
LPS-assembly protein LptD	lptD	R4YBZ4	85 kDa	OM	M			0.01023447	0.471236646	0.883737772	0.491339369
FhuA protein	fhuA	R4Y5U8	81 kDa	OM		P		0.058798537	0.279868452	0.019594643	0.451572495
Scaffolding protein for murein-synthesising holoenzyme	mipA	G0GL03	28 kDa	OM	M			0.137502918	0.002618601	0.208084223	0.08573534
OmpW protein	ompW	R4YFW6	23 kDa	OM	M			0.708077095	0.258341975	0.652891377	0.456906134
Outer membrane protein assembly factor BamB	yfgL	G0GPX6	42 kDa	OM			S	0.192957266	0.607182655	0.73626647	0.814302958
ScrY protein	scrY	R4YDP8	56 kDa	OM		G		0.539203585	0.368647318	0.987037329	0.45539581
Putative outer membrane lipoprotein	KPN2242_2257	G0GN82	22 kDa	OM	M			0.175531191	0.545685061	0.478859978	0.762660875
Outer membrane protein assembly factor BamC	bamC	R4Y768	37 kDa	OM			S	0.348473668	0.227055335	0.140293816	0.589196556
Penicillin-binding protein activator LpoA	lpoA	R4YEQ9	77 kDa	OM	M			0.052489047	0.174996986	0.355529362	0.4167415
OmpF protein	ompK35	O87753	40 kDa	OM	M			0.533894249	0.172492227	0.40607487	0.313537249
Vitamin B12 transporter BtuB	btuB	R4Y432	68 kDa	OM		H		0.014701073	0.067343206	0.016853675	0.132027758
YbaY protein	ybaY	R4YCW8	15 kDa	OM			S	0.702471586	0.648718487	0.554843651	0.756316846
HemX protein	hemX	R4Y7E1	43 kDa	OM		H		0.48715791	0.528714103	0.886212464	0.304681689
ABC transporter outer membrane lipoprotein	vacJ	G0GNY0	28 kDa	OM	M			0.04065466	0.285973299	0.632111876	0.537148005
UTP--glucose-1-phosphate uridylyltransferase subunit GalU	galU	G0GHM2	33 kDa	Cyt	M			0.034347097	0.59485965	0.20551062	0.025855205
UDP-glucose pyrophosphorylase	galF	A2V7R3	33 kDa	Cyt	M			0.119816487	0.04695553	0.484091117	0.042847711
60 kDa chaperonin groEL	groEL	G0GGA7	57 kDa	Cyt	O			0.947970127	0.5770576	0.263385196	0.014366737
Chaperone protein DnaK	dnaK	R4Y5P6	68 kDa	Cyt	O			0.08979587	0.975414471	0.835305306	0.072418572
FKBP-type peptidyl-prolyl cis-trans isomeraseTrigger factor	tig	R4YHL8	48 kDa	Cyt	O			0.938021264	0.89919677	0.458743457	0.390130434
Heat shock protein 90	htpG	G0GQB6	71 kDa	Cyt	O			0.078721774	0.87735271	0.798887446	0.155240973
ClpB protein	clpB	R4Y6I5	96 kDa	Cyt	O			0.943235249	0.009419364	0.875604728	0.043535515
Alkyl hydroperoxide reductase subunit C	ahpC	G0GT45	21 kDa	Cyt	O			0.030946765	0.278882194	0.326952871	0.762757251
Lon protease	lon	R4YEB6	89 kDa	Cyt	O			0.515628483	0.002942016	0.109416868	0.210164859
DegP protein	degP	R4Y5J4	50 kDa	PP	O			0.009792814	0.466458199	0.505534487	0.620710066
alkyl hydroperoxide reductase subunit C	KPR_4367	R4YIQ4	22 kDa	Cyt	O			0.013423263	0.000127981	0.689981828	0.117892572
Co-chaperonin GroES	groES1	G0GGA6	10 kDa	Cyt	O			0.09773809	0.371441019	0.246405738	0.373900966
peptidyl-prolyl cis-trans isomerase	ybaU	R4YHL6	68 kDa	PP	O			0.483881998	0.665568924	0.176606933	0.589196556
FtsH protease regulator HflK	hflK	G0GGE4	46 kDa	IM	O			0.804493398	0.208023667	0.912620939	0.223690157
Peptidyl-prolyl cis-trans isomerase	KPR_0740	R4YBB0	23 kDa	PP	O			0.061446496	0.328361551	0.820425116	0.459798732

Vita

Bethaney Cahill obtained a degree in Biology from the University of Tampa in 2011. She started the Master program at UNF in fall of 2012. Bethaney has spent the last two years working in a pathogenic bacteriology lab at UNF working on her thesis research. She will be receiving her Master's degree in Biology from the University of North Florida and will continue on to a PhD program in hopes of one day being a microbiology professor.