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Loss of outer membrane porins in clonally related clinical isolates of Klebsiella pneumoniae modifies the bacteria; resulting in altered resistance to phagocytosis by macrophages

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Loss of outer membrane porins in clonally related clinical isolates of *Klebsiella pneumoniae* modifies the bacteria; resulting in altered resistance to phagocytosis by macrophages

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DEDICATION

I would like to thank my wonderful parents for raising me to be as independent, self-sufficient, and strong willed as I am. Without that I would never have made it through. To my loving and supportive husband, Keenan, you have continuously inspired me to pursue my dreams. Your love and support have meant the world to me during this journey.

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Abstract:

Klebsiella pneumoniae is an opportunistic pathogen responsible for lobar pneumoniae, liver abscess, and septicemia. Clinical isolates are found to be extended spectrum beta lactamase positive with differential expression of the two classical porins, OmpK35 and OmpK36. Porin loss is associated with increased minimum inhibitory concentrations of beta lactam, cephalosporin, and carbapenem antibiotics that target the peptidoglycan. However, little is known about how porin loss affects other aspects of the cell envelope. The focus of this study was to characterize clinical isolates exhibiting differential porin expression and determine if the cumulative changes altered the resistance to phagocytosis by macrophages. The results support the hypothesis that porin loss significantly impacts the overall cell envelope composition, which in turn alters interactions with macrophages.

Chapter 1

Introduction

Outer membrane composition and permeability can lead to increased antibiotic resistance

Gram-negative bacteria are distinguished as having both an inner and an outer plasma membrane that is separated by a thin layer of peptidoglycan within the periplasmic space (**Figure 1)**. This creates a stable cell envelope with two selectively permeable membranes that separate the inside of the cell from the environment. Maintenance of the cell envelope is crucial to the survival of the organism. The outer membrane is chemically distinct from the inner membrane. The phospholipids of the outer leaflet are modified and are known as the immunogenic lipopolysaccharide (LPS). It is also known as endotoxin due to its toxic nature in mammalian hosts and interactions with LPS and host immune cells have been well documented [1–3].

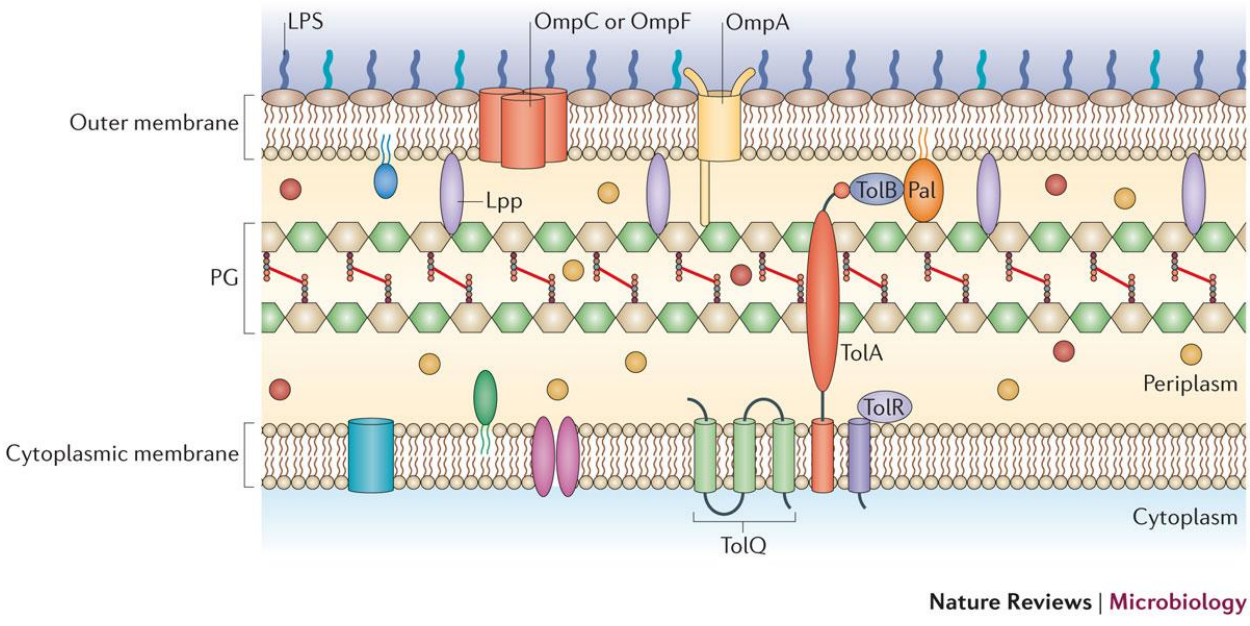


Figure 1. Gram-negative bacteria cell envelope consists of an inner and outer plasma membrane separated by a thin layer of peptidoglycan. The outer leaflet of the outer membrane consists of lipopolysaccharide (LPS) and is surrounded by capsular polysaccharide. Embedded within the outer membrane are proteins including porins that allow the passive diffusion of small molecules into the periplasmic space[4].

The other major components of the outer membrane encountered by host immune cells are outer membrane proteins which are potentially important virulence factors. The functions of these proteins include adherence to surfaces, cell to cell communication, and transport of molecules across the membrane. Non-specific transport of molecules from the exterior environment to the periplasmic space is handled by outer membrane proteins that create pores in the membrane and are thus called outer membrane porins[5–9]. Porins have recently been shown to also be important to interactions between the bacteria and the host immune system, particularly the evasion of phagocytosis[2,10].

Major, common porins OmpC and OmpF have been well characterized in *Escherichia coli*[9,11]. These porins have several homologues in various Gram-negative species, including *Klebsiella pneumoniae* which are denoted as OmpK36 and OmpK35 respectively[7,8]. Porins are beta barrel proteins that form a homotrimer in the outer membrane (**Figure 2)**. This creates a central pore that allows for the passive diffusion of small hydrophilic molecules into the periplasmic space.

OmpK35 and OmpK36 are both non-specific porins of slightly different molecular weight (35kDa and 36kDa respectively). The two major porins are highly similar in function, but have been shown to differ in permeability. In *E. coli,* OmpF (OmpK35 homologue) has a higher rate of influx for large solutes and is in general more permeable than OmpC (OmpK36 homologue). Using liposome swelling assays, OmpC was shown to have 15-20% of the permeability to β-lactam antibiotics compared to OmpF. However, OmpC also retained 50% of the permeability for small molecules like glucose[12]. Based on sequence homology, crystal structures, and permeability to β-lactam antibiotics; it is assumed that the permeability of OmpK35 and OmpK36 of *K. pneumoniae* are similar to their *E. coli* homologues[7,8,13,14]. X-ray crystallography structures of both porins have

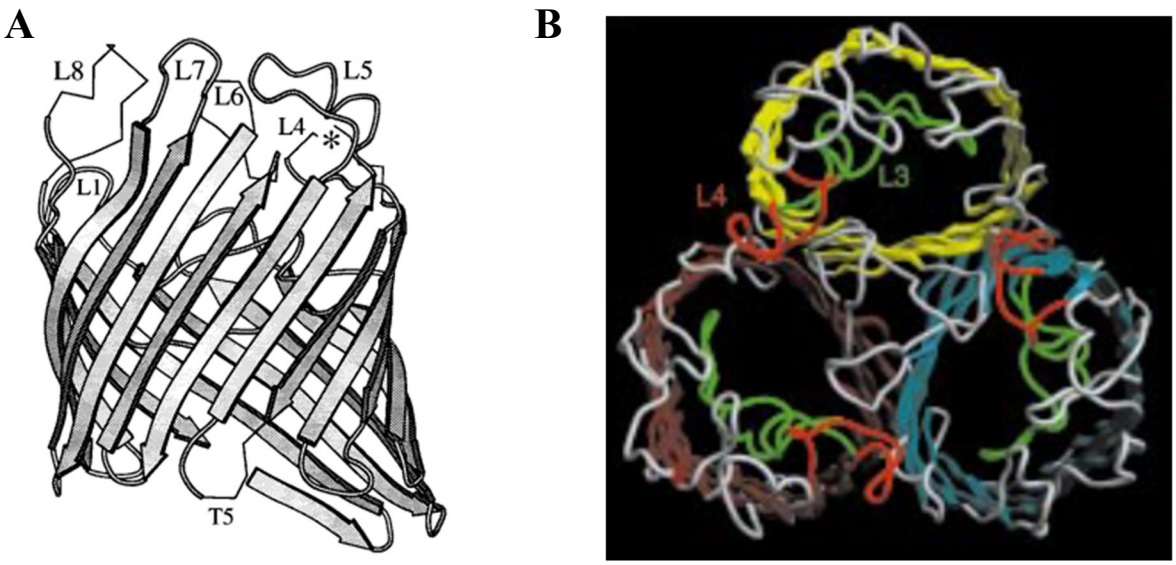


Figure 2. OmpK35 and OmpK36 are β-barrel proteins that form a homotrimer within the outer membrane. This creates a central pore through which small molecules can traverse the outer membrane. A) Proposed structure of OmpK36 monomeric subunit[7]. B) 3D modeling of OmpK36 homotrimer featuring the central pore[8].

shown a similar pore geometry. Interestingly, it was shown that the increase in charge density of OmpK36 in comparison to OmpK35, and not pore size, was responsible for the slower influx rates of solutes[8,12].

This difference in the rate of influx of molecules from the exterior of the cell to the periplasmic space between OmpK35 and OmpK36 could be an evolutionary advantage for the expression of one porin over the other in contrasting conditions[15]. While porins play an important role in nutrient acquisition[6,16] the nonspecific nature of these porins also allows the passage of small antibiotics as well[6,7,12]. It has been shown in *K. pneumoniae* that loss of one or both porins can have a dramatic increase on the organism's resistance to β-lactam antibiotics. This is because the target of these antibiotics is the peptidoglycan found in the periplasmic space between the two membranes[5,17–19].

It has been noted during investigations into several clinical outbreaks that decreased outer membrane porin expression is correlated with an increased minimum inhibitory concentration (MIC) to multiple classes of antibiotics. More specifically, loss of one or both porins is often observed in many clinical isolates that can be classified as Extended Spectrum Beta Lactamase (ESBL) positive or multidrug resistant (MDR)[5,15,20–22]. Extended spectrum beta lactamase enzymes target most β-lactam antibiotics including penicillin and cephalosporin, neutralizing the antibiotic[5].

Surveillance studies on antibiotic resistant clinical isolates have shown that in *Klebsiella pneumoniae*, and other enteric species, most ESBL producing isolates have some form of porin loss. It is more likely to observe the loss of OmpK35 or both porins than it is to observe the loss of only OmpK36. However, ESBL negative bacteria typically express both porins[5,17,23–25]. In one specific study using 52 clinical isolates from a hospital outbreak in Iran, it was shown that 30

isolates were ESBL negative and maintained expression of both porins. However, of the 22 ESBL positive isolates, 9 were deficient in OmpK35 only, 4 were deficient in OmpK36 only, and 5 of the isolates were deficient in both porins[26]. Another study using 57 carbapenem resistant isolates (KPC) showed that dual porin loss acted synergistically with carbapenemase to increase resistance[17], and a third showed that of 12 KPC producing isolates, there was no detectable expression of *OmpK35*[24]. Although OmpK36 mutants have been identified in *K. pneumoniae,* it still seems apparent that loss of OmpK35 or both porins is more common in antibiotic resistant clinical isolates than loss of OmpK36 alone[6,17,24,27]. This is most likely due to the fact that OmpK35 is transcriptionally depressed in environments similar to those found in a human host as well as when exposed to antibiotics *in vitro*[6,24,26,28].

Infections classified as ESBL positive or MDR caused by Gram-negative bacteria are common place in hospitals and clinics throughout the world[23,29]. The rapid adaptation of bacteria to environmental pressures, including antibiotics and the harsh conditions of the mammalian gut, have resulted in many alterations to the Gram-negative cell envelope[30]. This includes loss of one or both of the major porins, OmpK35 and OmpK36[5]. Expression of the porins is tightly regulated by multiple environmental response pathways that can be activated by antibiotic stress[5,6,30].

Regulation of OmpK35 and OmpK36

Regulation of OmpK35, OmpK36, and other outer membrane proteins occurs through the coordinated efforts of several regulatory systems. This includes the major two-component stress response regulators EnvZ/OmpR[31,32], and CpxA-CpxR[28,32,33]. Extensive research on the regulation of each of these systems has revealed complex pathways that result in transcriptional and post transcriptional regulation of the porins, resulting in the dominant expression of one porin over the other, depending on the environmental stressors present.

EnvZ/OmpR regulation of OmpC and OmpF is based on promoter affinity for the transcriptional regulator OmpR (**Figure 3)**[34–36]. The promoter for *ompF* has a high affinity for OmpR and requires lower concentrations to activate transcription. Conversely, the *ompC* promoter has a low affinity for OmpR and requires increased concentrations to activate transcription[35]. The environmental sensor kinase Env*Z* is activated by increased osmolality, higher temperatures, and low pH[37]. Activation of EnvZ results in the phosphorylation and activation of OmpR, and an increased transcription of both *ompC* and a small inhibitory RNA. This RNA, *micF*, binds to the 5` end of the *ompF* transcript and depresses translation[35]. There is also evidence to support the hypothesis that at increased levels of activated OmpR, a low affinity binding site close to the *ompF* promoter binds OmpR and causes the DNA to fold into a hairpin loop and block transcription of the porin.[35] This allows for the differential expression of OmpF over OmpC in environments low in nutrient availability, neutral pH, and low temperature like those of the natural environment. However, in environments similar to those of the host, with high nutrient availability, lower pH, and increased temperature, OmpC becomes the dominant porin[31,35].

The two component CpxA-CpxR envelope stress response system also differentially regulates the expression of OmpK35 and OmpK36. Similarly, to EnvZ/OmpR, CpxA serves as a membrane associated sensor kinase and CpxR acts as the regulator of transcription. It has been shown that CpxA is activated by stress to the outer membrane to include osmolality, pH, surface adhesion, detergents, and presence of misfolded proteins. Activated CpxA in turn begins a phosphorylation cascade ultimately resulting in phosphorylation of CpxR (CpxR-P), which then regulates transcription of target genes. Overexpression of CpxA has been shown to increase the transcriptional expression of *OmpK36* and significantly decrease *OmpK35* expression in *K. pneumoniae*[33].

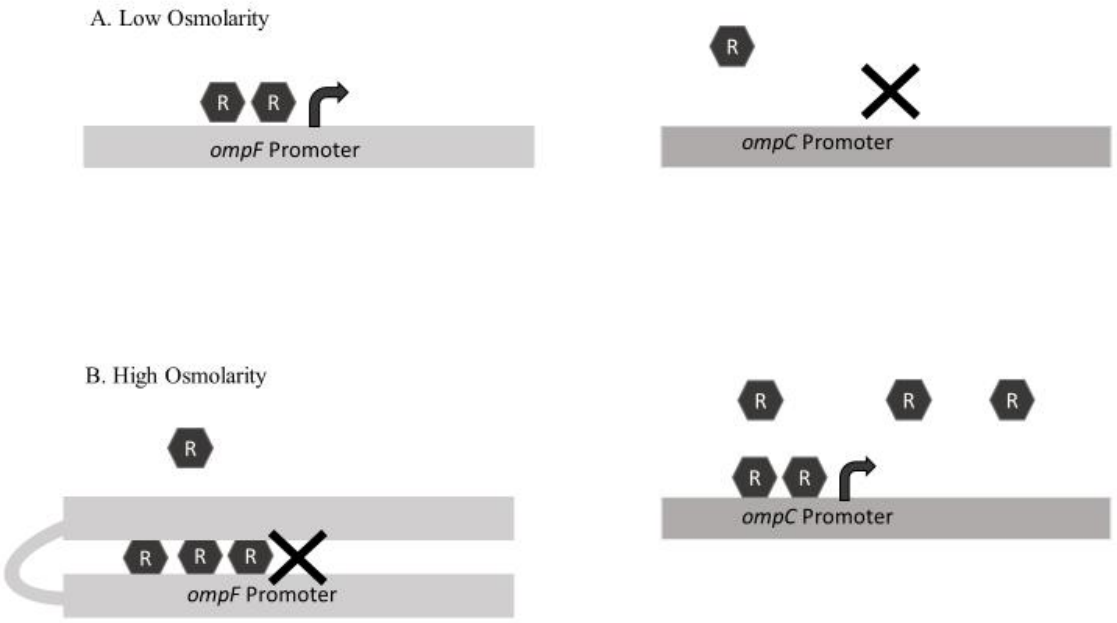


Figure 3. EnvZ/OmpR regulation of *ompC/ompF* transcription occurs through promoter affinity for the transcriptional regulator, OmpR. Image is based on previous work by Mattison *et al*[38]. A) In low osmolarity conditions OmpR levels are low. The high affinity *ompF* promoter binds available OmpR and the low affinity *ompC* promoter does not. This promotes transcription of *ompF* over *ompC.* B) In high osmolarity conditions, the high affinity *ompF* promoter and an upstream response regulator bind OmpR, causing a bend in the DNA and blocks transcription of *ompF.* However, the low affinity *ompC* promoter binds OmpR and activates transcription of *ompC.*

CpxA-CpxR has also been shown to be required for *K. pneumoniae* survival in environments that mimic the host gastrointestinal tract (GI). This regulatory system is important in environments containing bile salts, high NaCl concentration, oxidative stressors (hydrogen peroxide), or disinfectants (chlorohexidine). When CpxA-CpxR was knocked out, survival in the presence of these materials decreased significantly[28]. This is fundamentally important for pathogenicity within a host as colonization within the GI tract is required to establish a *K. pneumoniae* infection.[39] It was also shown that this stress response system was important for antibiotic resistance as minimum inhibitory concentrations of several β-lactam antibiotics were decreased in *cpxA-cpxR* mutants. Notably, this same study also concluded that the promoter region of *OmpK36* contains a conserved CpxR binding motif that is a transcriptional regulatory site[28]. Collectively, these studies indicate the importance of porin regulation in the host environment and add to the evidence that OmpK36 is an important virulence factor.

This dual regulation of *OmpK35* and *OmpK36* may have arisen from the evolutionary advantage of expressing one porin over the other under contrasting conditions as the two porins allow for different rates of diffusion across the outer membrane[8,12]. The central pore of OmpK35 is notably more permeable than that of OmpK36 and may be more beneficial for nutrient acquisition in environments naturally low in salt and carbon sources[6,8,12]. However, the increased rate of influx of molecules would be detrimental in environments like the human gut where toxic components (like bile salts/ proteolytic enzymes/ antibiotics) may be more abundant. Therefore, it is likely more constructive for the bacteria to down regulate *OmpK35* expression and upregulate *OmpK36* as the OmpK36 central pore is less permeable than that of OmpK35. This difference in pore permeability may decrease the influx of larger molecules that may be harmful to the cell while still allowing passage of small nutritive molecules like glucose[5,7,12,17].

Porin loss can change the cell surface by impacting expression of other outer membrane proteins

Porin loss does not only affect the permeability of the outer membrane. Expression of other outer membrane proteins could also be affected. Specifically, proteins that are involved in nutrient acquisition, adherence, and outer membrane stability have the greatest potential to be affected. OmpK26, LamB, OmpA, and Lpp have been determined to be important in the maintenance of the cell envelope.

OmpK26 is a 26kDa porin similar in structure to OmpK35 and OmpK36[40]. However, this porin is specific for oligo galacturonate and will not allow the passage of antibiotics. Under normal conditions, expression of this porin is inactive and does not appear on an SDS-PAGE gel. However, OmpK26 was shown to be essential for survival in an OmpK36 deficient strain. Expression of this porin in the absence of OmpK36, along with the presence of beta lactamase, confers resistance to carbapenem and β-lactam antibiotics *in vitro*. This also causes a decrease in the virulence of the organism in a mouse model[40].

LamB is a maltose-specific porin that is permeable to β-lactam antibiotics, but to a lesser degree than OmpK35 or OmpK36[41]. It is suspected to be significantly upregulated with dual porin loss. LamB has been shown to be. In a study using two clinical isolates, one expressing only OmpK36 and the other expressing neither porin, transcript level of *lamB* was 2 fold higher in the dual porin loss isolate[41]. However, transcriptional expression of *lamB* has yet to be determined in comparison to a strain expressing only OmpK35 or both porins.

The outer membrane protein OmpA has shown to be multifunctional and important for outer membrane stability, pathogenicity, and antimicrobial resistance. The main function of OmpA

is to link the outer membrane and peptidoglycan via a C-terminal peptidoglycan binding domain. Linkage of the outer membrane to the peptidoglycan by OmpA has shown to be important to resistance of tensile stress on the peptidoglycan and support overall integrity of the cell envelope[42]. Mounting evidence also suggests OmpA acts as an adhesin and is important for invasion into epithelial cells and phagocytes[43,44]. The progression of infection to bacteremia and meningitis infections *in vivo* are also OmpA dependent [2,45]. In *E. coli,* OmpA deletion showed an increase in OmpC on an SDS-PAGE gel[46]. *K. pneumoniae* outer membrane vesicles also showed increased OmpA expression when OmpK35 is lost[47]. However, the change in transcriptional expression of OmpA due to porin loss has yet to be determined.

Murein (peptidoglycan) lipoprotein (Lpp) is highly abundant within the outer membrane and serves to stabilize the cell envelope. The fatty acid moiety is embedded in the outer membrane and the protein is also covalently linked to the peptidoglycan. Lpp mutants have been shown to increase production of outer membrane vesicles and leak periplasmic enzymes. This indicates the importance of Lpp to the outer membrane and the stability of the cell envelope[6,27,48]. Lpp has also been indicated in pathogenesis of bacterial infection because Lpp mutants are more susceptible to serum killing and phagocytosis *in vitro*[48]. Although Lpp is essential for membrane stability, no correlation between porin loss and Lpp expression has been investigated.

Capsular polysaccharide may be altered due to porin loss

Capsular polysaccharide is another important virulence factor for *K. pneumoniae*. It is composed of a poly-KDO linker moiety that is associated with the outer membrane, and a K-antigen repeating polysaccharide unit that is serotype specific[49]. There are approximately 77 known *K. pneumoniae* capsular serotypes that are highly suggestive of pathogenicity. K1-K6 are more often associated with respiratory infections and septicemia than others[50]. Serotypes K1 and

K2 are the most common culprits for invasive infections[51] and K2 is the most prevalent serotype of clinical isolates[52].

All *K. pneumoniae* serotypes contain the monosaccharide glucuronic acid as a component of its K-antigen polysaccharide[49]. The amount of glucuronic acid within a sample can be quantified and used as an indirect method of determining capsule production[53]. Srinivasan *et al.* 2012 showed that a *K. pneumoniae ompK36* knockouts from strain NTUH K2044 of K1 serotype had a significantly decreased production of glucuronic acid compared to the wildtype[25]. Conversely, Tsai *et al.* 2011 noted a more mucoid appearance of colonies indicative of increased capsule production for *ompK36* mutants for *K. pneumoniae* NVT1001 of K2 serotype[10]. This alteration to capsule production due to porin loss could potentially impact the bacterial resistance to phagocytosis by macrophages *in vitro*[2] and requires further investigation as it may be serotype dependent.

Klebsiella pneumoniae **pathology**

Since porins are surface antigens that interact with immune cells, loss of either porin due to environmental selective pressures can ultimately result in altered interactions with the immune system. Before this can be considered, it is important to understand normal pathology for *K. pneumoniae*.

Klebsiella pneumoniae is an enteric bacterium that is also an opportunistic pathogen. Initial colonization of the gut is an important step in *K. pneumoniae* pathogenesis that is required for the establishment of other infections[39]. This includes pneumonias, liver abscesses, urinary tract infections, and bacteremia. *Klebsiella pneumoniae* is part of a group of bacteria referred to as the ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumanii, Pseudomonas aeruginosa and Enterobacter* species)[54].

These bacteria are known for being highly adaptable to environmental pressures. This is especially true for rapid adaptation to antibiotics and the immune system during respiratory infection[54,55].

Klebsiella pneumoniae possesses numerous virulence factors that have developed during co-evolution alongside the host innate and adaptive immune systems[18,54,56]. This includes production of a protective layer of capsular polysaccharide, referred to as the capsule. The capsule is essentially a slimy layer that surrounds the bacteria, making the bacteria difficult for professional phagocytes to ingest[50,57]. It can also act as a protective layer against complement-mediated killing by masking other bacterial surface antigens like the LPS or outer membrane proteins that trigger the complement cascade *in vitro*.[58] Certain serotypes of capsule can be especially dangerous as they incorporate sugars, like sialic acid, that are typically expressed by mammalian cells. The expression of sialic acid in the capsule can then be recognized by the immune system as "self" and will not be targeted as a pathogenic invader[59].

Colonization of epithelial cells or indwelling abiotic surfaces, such as catheters, is an important step in *K. pneumoniae* pathology[54,55]. Studies have shown that for *K. pneumoniae* infections to persist, colonization of the gut is also necessary[39]. Adherence to surface, whether epithelial lining of the lung, gut, or a catheter, is dependent on bacterial proteins that anchor the bacteria to the surface. This attachment must be strong enough to withstand mechanical forces such as coughing, blood flow, and peristalsis[23,39,55]. Fimbriae are the proteins that are primarily responsible for this attachment in *K. pneumoniae*[54,60]. The outer membrane protein OmpA has also been indicated as an adhesin involved in attachment and invasion of epithelium[2,44,45].

Like most Gram-negative bacteria, *K. pneumoniae* is also adept at evading the inflammatory response. Lipopolysaccharide is a highly immunogenic molecule that activates

toll-like receptor 4 (TLR-4) and elicits a strong inflammatory response[55]. However, *K. pneumoniae* can avoid this activation by genetic switching of antigenic determinants within the LPS. Attenuated inflammatory responses can allow for the establishment and maintenance of more severe infections LPS [1,2,58,61].

Evasion of the innate immune system and attenuation of the inflammatory response are two of the most efficient tactics for the successful infection of the host used by *K. pneumoniae*[23,54,56]. Recent studies have focused on understanding the mechanisms involved. *Frank et al. 2013* showed *K. pneumoniae* targets a PI3-kinase signaling pathway that blocks the transcription factor NF-κB from entering the nucleus to mediate the inflammatory response of A549 lung epithelial cells *in vitro*. The suppression of the inflammatory response was dependent of capsule production, as capsule mutants did invoke an inflammatory response[55].

Another study from the same lab showed that *K. pneumoniae* survives phagocytosis by macrophages by activation of another PI3-kinase signaling pathway that causes the subversion of phago-lysosomal maturation and ultimately results in induction of apoptosis *in vitro*. The bacteria can then escape the macrophage during apoptosis and begin to proliferate extracellularly. This was not dependent on capsule production, however, as capsule mutants were still able to survive within macrophages *in vitro*[62].

Clinical studies have also revealed an emergence of hypervirulent mucoviscous *K. pneumoniae*[56] as well as an increase in the prevalence of carbapenem resistant *K. pneumoniae* (KPC)[18,54]. The current hypothesis is that the rapid adaptation to antibiotics and to the innate immune system together have resulted in a more dangerous pathogen[18,56,63]. This is especially true in areas with unrestricted use of antibiotics as community acquired infections in healthy children are becoming more prevalent[64]. This pathogen is constantly adapting and evolving to

environmental pressures, making a full understanding of its interactions with the immune system both difficult and necessary.

Vaccine potential for OmpK35 and OmpK36

Multiple studies have shown the potential for OmpK35 and OmpK36 as vaccine candidates, because both porins elicit an immune response that confers protection against lethal challenge of live bacteria *in vivo*[10,65]. Research looking at the vaccine potential for both OmpK35 and OmpK36 homologues span across multiple Gram-negative species including *E. coli, K. pneumoniae, Salmonella, Shigella, Aeromonas hydrophila, Psuedomonas aeruginosa* and *Vibrio cholera*[15,65–72].

To assess the immunogenicity of OmpC and OmpF *in vivo*, Liu *et al.* 2012 injected purified recombinant OmpF and OmpC into mice and measured the IgG1 and IgG2 antibody titers. After both the first and second injections of recombinant protein, IgG1 and IgG2 titers were higher in both OmpF and OmpC treated mice than in the adjuvant injected mice. OmpF had higher IgG1 and IgG2 titers than OmpC, and both OmpF and OmpC showed higher titers for IgG1 over IgG2. This indicates that both OmpF and OmpC can produce a mixed cell-mediated and humoral immune response. Mice immunized with the recombinant proteins were protected against lethal challenge with live bacteria as well, making the case for OmpC and OmpF as vaccine candidates.[15]

Interestingly, in contrast to other Gram-negative enteric species, OmpK36 DNA vaccines from *K. pneumoniae* promote a pro-inflammatory Th1 immune response over a Th2 response *in vivo*, indicating a cell-mediated response. However, Th2 indicators were also increased during immunization with OmpK36 so a humoral response is also possible. Immune sera containing anti-OmpK36 antibodies increased opsonophagocytic killing assays *in vitro* (95.2% intradermal

administration, 82.4% intramuscular administration) and mice immunized against OmpK36 also conferred 100% protection via intradermal vaccination *in vivo*[65].

Collectively, these studies show immune cell recognition of recombinant OmpC, OmpF and OmpK36 and the ability of these proteins to induce an immune response. The vaccine potential of the porins is increased by the protective effect vaccination with recombinant porins had on lethal challenge *in vivo*. However, ESBL positive clinical isolates that are both resistant to antibiotics and pose a threat in a hospital setting are likely to exhibit porin loss to some degree. Therefore, it is important to investigate how loss of one or both porins will alter the bacterial interaction with the host.

Porin loss impact host-pathogen interactions

Porin loss in clinical isolates is a common phenomenon and significantly changes the way the bacteria interact with cells of the immune system as seen in many Gram-negative opportunistic pathogens. Isogenic porin knockouts created in the lab show a correlation of porin loss with a decrease in overall virulence both *in vitro* and *in vivo*[2,10,20]. Porin loss alters the way the pathogen interacts with phagocytic cells, activates the classic complement pathway, elicits an immune response, and reduces the ability of the bacteria to cause fatal infection in a mouse model[2,10,47,73].

Complement mediated killing of *K. pneumoniae* begins with the binding of OmpK36 to the C1q complement protein. This activates the classic complement pathway and results in destruction of the bacteria. Alberti *et al.* 1993 showed that outer membrane porins (OmpK36 in particular) but no other outer membrane components (LPS or CPS) activate this particular pathway in human sera *in vitro* that leads bacterial killing[13]. In a similar study involving *E. coli* it was shown that loss of the OmpC porin lead to increased survival in human serum *in vitro* by evading

activation of the antibody dependent classic complement pathway. All assays using OmpC deficient *E. coli* were inefficient at bacterial killing. This demonstrates the possible ability of clinical isolates without OmpC expression to evade complement mediated killing in the host[74].

In *K. pneumoniae* multiple independent studies have shown that loss of OmpK36 results in a decreased resistance to phagocytosis. Tsai *et al.* 2011 used neutrophil phagocytosis assays *in vitro* to determine the percent phagocytosis of OmpK35 and OmpK36 isogenic mutants and observed lethal effects of the bacteria in a peritonitis mouse model *in vivo*. The results concluded that loss of OmpK36 or both porins significantly reduced phagocytic resistance to neutrophils. Interestingly, loss of only OmpK36 (compared to the wildtype) resulted in a significantly increased survival time and slightly decreased death rate (10% survival compared to 0% survival by mice infected with wildtype); while loss of only OmpK35 had no effect on lethality. However, loss of both porins resulted in a significantly increased survival time and decreased death rate of the mice (50% compared to 0 %)[10]. This indicates an overall importance of OmpK36 as a virulence factor with a lesser emphasis on OmpK35.

Importance of OmpK36 in *K. pneumoniae* pathogenesis was also discussed by March *et al*. 2013. Both *Dictyostelium discoideum* (an amoeba) and murine macrophages demonstrated increased phagocytosis of bacteria lacking OmpK36 as opposed to those expressing it *in vitro*. This study also looked at the role of OmpK36 in infection and dissemination *in vivo* in a mouse model. Mice were infected intranasally and 24 or 72 hours post infection the mice were sacrificed. Bacterial load in peripheral organs was used to determine dissemination. Although the mutants could colonize the trachea and cause infection in the lungs, bacterial load in the lungs was significantly decreased after 72 hours post infection. The mutants were also able to migrate to the spleen and liver but were significantly lower in bacterial load after 72 hours post infection[2]. This

study made it clear that although porin loss does decrease virulence, porin loss isolates would still be able to establish an infection in the host and pose a problem for immune compromised individuals.

Klebsiella pneumoniae OmpK36 has also been indicated as being potentially important for survival in the harsh conditions of the host environment. When bacterial growth was examined *in vitro* in the presence of increasing concentrations of bile salts, hydrogen peroxide (reactive oxygen species), and two different reactive nitrogen species it was evident that loss of OmpK36 resulted in a decrease in survival. As these conditions mimic those found in a host (gut or phagolysosome) this study pointed out possible reasons for decreased virulence and pathogenicity of OmpK36 mutants observed when incubated with *Caenorhabdits elegans* infection model *in vivo*[25].

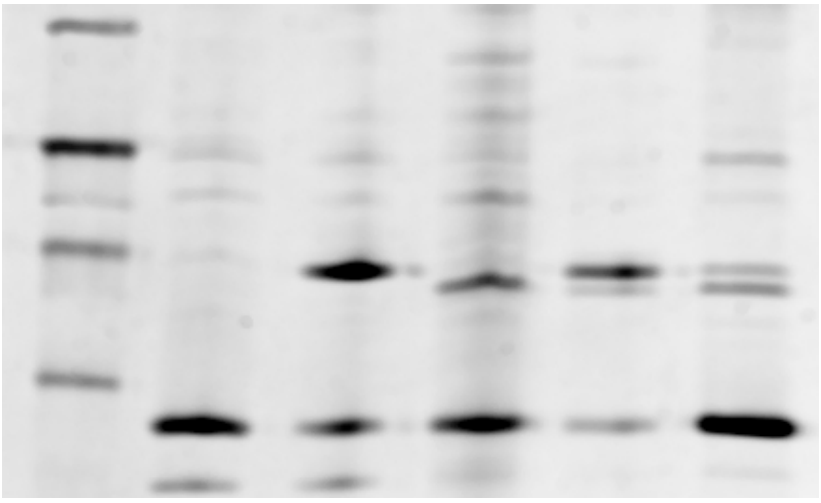
All Gram-negative species of bacteria secrete outer membrane vesicles (OMVs), the contents of which mimic that of the outer membrane. *Klebsiella pneumoniae* OMVs purified from strains exhibiting differential expression of OmpK35 and OmpK36 elicited unique immune responses in a macrophage tissue culture model *in vitro*. Loss of OmpK36 or both porins showed a significant decrease in production of Mip2 which is important for the chemotaxis of neutrophils. Dual porin loss also resulted in significant depression of the TNF-α inflammatory response. These data indicate a significant impact on the immune response, particularly for strains exhibiting loss of both porins, that may result in suppression of the inflammatory response[47].

Thesis Aim

Porin loss in *Klebsiella pneumoniae* is a clinically important phenomenon that has been shown to alter both antibiotic resistance as well as pathogenicity of the organism. There is a lack of information surrounding the impact porin loss has on transcriptional expression of other outer

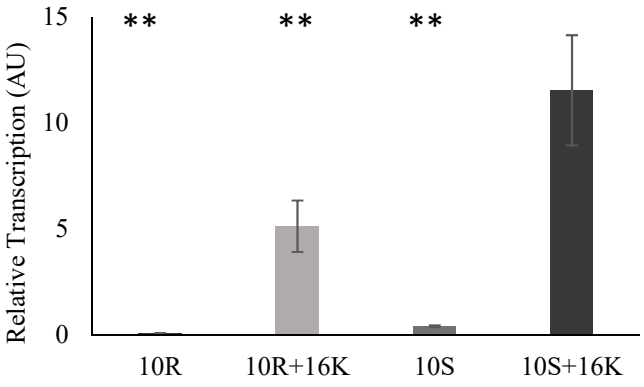
membrane proteins, capsule production, and LPS content. These factors have been previously implicated as important virulence factors, therefore alterations due to porin loss could contribute to the diminished pathogenicity previously observed. Therefore, the hypothesis is that loss of OmpK35 and/or OmpK36 will alter the cell surface and result in altered resistance to phagocytosis by macrophages. The present study aimed to determine the downstream effects porin loss had on other aspects of the outer membrane. Utilizing clonally related clinical isolates exhibiting differential porin expression (**Figure 4A)**, the impact porin loss has on growth, capsule, LPS, and transcriptional expression of other outer membrane proteins was determined. These aspects were chosen as previous investigation has implicated that these components could potentially confer resistance or susceptibility to phagocytosis.

A. Protein gel



Porin Expression					
OmpK35	-	+	-	+	+
OmpK36	-	-	+	+	+

B. *ompK35*



C. *ompK36*

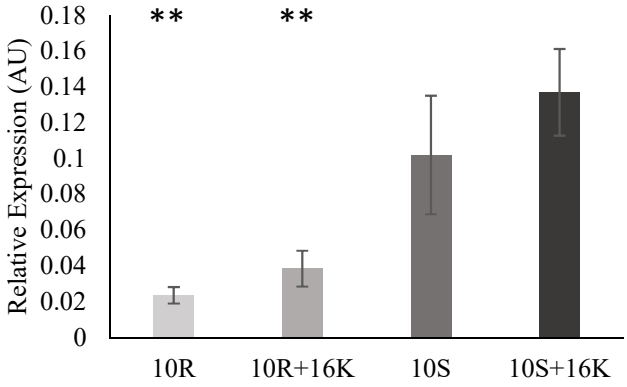


Figure 4. A) Outer membrane from each strain was purified and proteins were visualized on a 12% SDS-PAGE gel using SYPRO Ruby stain. Outer membrane profiles of clinical isolates exhibiting differential porin expression of OmpK35 and OmpK36 and a lab strain (ATCC 43816) used in this study. Transcriptional expression of outer membrane proteins by qPCR and analyzed using the ΔΔCt method. Genes are normalized to *gapA* and clinical isolates are normalized to the lab strain 43816. B) *ompK35* and C) *ompK36* transcriptional expression corresponds to protein profiles of SDS-PAGE gels. *$p<0.01$ **$p<0.0001$ compared to 10S+16K $n\geq7$ from at least 2 independent cultures.

It was expected that porin loss would have an impact on the production of capsular polysaccharide. The assay showed that loss of a single porin decreased the overall amount of capsule produced, but loss of both porins increased capsule production. The amount of LPS was also expected to be altered by loss of one or both porins, which was also supported by the data. Previous study has indicated that loss of OmpK35 or OmpK36 has a significant impact the expression of other outer membrane porins in a compensatory fashion but there is insufficient evidence for the impact porin loss has on structural proteins. The present study showed that loss of one or both porins is compensated for by increased expression of oligo galacturonate specific *ompK26* or the maltoporin *lamB.* The expression of the structural proteins, *ompA* and *lpp,* were also modified in response to porin loss. Surprisingly, there was a significant impact on resistance to phagocytosis by loss of a single porin, but not both. Based on these findings and others we conclude that although porin loss has a large impact on the cell envelope of *K. pneumoniae*, these cumulative changes do not necessarily predict resistance to phagocytosis. However, other aspects of macrophage-pathogen interactions may be more affected by porin loss and requires further study.

Chapter 2

Porin loss affects the cell envelope of *Klebsiella pneumoniae* and overall resistance to phagocytosis by macrophages

Methods

Bacterial strains, plasmids, and media

Clonally related clinical isolates were cultured from a single patient during a clinical outbreak and were provided by Dr. Sebastian Alberti (University of the Balearic Islands, Palma de Mallorca, Spain). Isolates CSUB10R (10R) expressed neither porin and CSUB10S (10S) expressed only OmpK36[5]. A plasmid carrying the o*mpK35* gene (pSHA16K) was transformed into CSUB10R and CSUB10S to create CSUB10R+ pSHA16K (10R+16K) which expressed only OmpK35 and CSUB10S + pSHA16K (10S+16K) which expressed both porins. These clinical isolates have been previously characterized as extended spectrum β-lactamase positive (ESBL) and have high minimum inhibitory concentrations for several β-lactam antibiotics[5]. All isolates were grown in Luria-Bertani broth at 37°C with 16μg/mL cephalothin to maintain the antibiotic selective pressure present when isolated. Isolates 10R+16K and 10S+16K were also grown with 50μg/mL kanamycin for plasmid maintenance.

The lab strain ATCC 43816[75] contains no known antibiotic resistance plasmids and has been characterized as ESBL negative. This strain was used as a basis for comparison of the clinical isolates against a less antibiotic resistant and virulent strain expressing both porins. It was grown in Luria-Bertani broth at 37°C.

Growth

Growth curves were performed by inoculating 50mL of fresh Luria-Bertani broth (supplemented with antibiotics when appropriate) with a 1:1000 dilution of overnight culture. The OD_{600} was determined using an Eppendorf Bio photometer every 30 minutes for 6.5 hours.

Overnight cultures (approximately 15 hours) were serially diluted and plated onto Luria-Bertani agar plates and incubated at 37°C overnight. Colony forming units (CFUs) were counted and CFUs/mL were calculated to determine the effect porin loss has on the concentration of an overnight culture. Colony morphology was visualized by streak plating onto Luria-Bertani agar plates. The plates were observed approximately 15 hours after plating.

Outer membrane isolation

The outer membrane was isolated using previously described methods [3]. Cultures were grown overnight; 1 mL of overnight culture was used to inoculate 100mL of fresh Luria-Bertani with the appropriate antibiotic and grown to an OD_{600} between 1.5-2.0. Cultures were then centrifuged at 10,000xg for 10 minutes. The cell pellet was resuspended in Tris-sucrose solution (20mM Tris, 20% sucrose, pH 8.0). The cell wall was digested using lysozyme (15mg/mL) in 0.1M EDTA solution for 40 minutes and then 0.5M $MgCl_2$ was added to the lysate. The mixture was centrifuged for 20 minutes at 12,000xg and the supernatant decanted. The pellet was dissolved in 10mM Tris (pH 8.0) and sonicated on ice. Cellular debris was pelleted and the supernatant containing the membranes was further centrifuged at 40,000xg for 60 minutes. The pellet was resuspended in DI water. The membrane fractions were treated with Sarkosyl solution at room temperature for 20 minutes and centrifuged at 40,000xg for 90 minutes. The pellet containing outer membrane was resuspended in 1X PBS. Protein concentration was determined using a standard Bradford assay (Coomaisse Plus, Thermofisher). Proteins were visualized on a 12% SDS-PAGE gel using the SYPRO Ruby stain from Molecular probes[76]. **Minimum Inhibitory Concentration**

Overnight cultures were diluted to a suspension containing approximately 10^3 CFUs/mL in LB media in a 96 well microtiter plate. Bacteria were treated with 500, 250, 125, 62.5, 31.25, 16, 8, 4, 2, 1, and .5 μg/mL of cephalothin, gentamicin, or colistin. Wells containing only LB were

used as controls for normal growth. The absorbance of each well at OD_{600} was measured using a Biotek Gen5 plate reader. The minimum inhibitory concentration was determined as the lowest concentration at which growth was significantly inhibited (by at least 90%) as defined by the Clinical and Laboratory Standards Institute (CLSI)[77].

Capsular polysaccharide characterization

Capsular polysaccharide (CPS) was extracted using the protocol outlined by Domineco *et al*[53]. From an overnight culture, 500µL was mixed with 100µL of 1% Zwittergent 3-14 in 100mM citric acid, pH 2.0. The mixture was vortexed vigorously, incubated at 50°C for 20 minutes, and centrifuged for 5 minutes at 14,000 rpm. The supernatant was transferred to a 2mL Eppendorf tube and mixed with 1.2mL of absolute ethanol and incubated for 90 minutes at 4°C. Precipitate was collected after centrifugation at 14,000 rpm for 10 minutes and then dissolved in 200µL DI water.

CPS was quantified following previously established protocol by Lin *et al*[78]. Samples were vortexed vigorously with 1.2mL of 12.5mM sodium tetraborate in concentrated sulfuric acid and heated for 5 minutes at 95°C. The samples were allowed to cool before the addition of 20µL of 0.15% m-hydroxydiphenyl. The absorbance at 540nm was measured with a Biotek Gen5 plate reader. A standard curve was generated using D-glucuronic acid in order to determine the concentration of glucuronic acid of the CPS samples. To ensure quantification of CPS from the same number of bacteria, CFUs/mL were determined and strains were normalized to 10^8 CFUs/mL. Each assay was performed in triplicate from 6 individual cultures.

To determine the molecular weight distribution of the capsule, a sample of the capsule containing 10µg of glucuronic acid was run on a 12% SDS-PAGE gel. Polysaccharide was visualized using the ProQ Emerald 300 staining kit by Molecular probes[79].

Capsule was stained using overnight cultures and a 10% nigrosin solution. An inoculation loop was used to add bacteria to the slide and mixed with 1µL of nigrosin. The solution was spread across the slide and allowed to air dry. The slide was visualized at 1000x total magnification with a compound light microscope.

Lipopolysaccharide Quantification

To quantify LPS of whole cell bacteria, a previously described purpald assay method was used[47,80]. This assay detects the 3-deoxy-D-manno-oct-2-ulopyranosonic acid (KDO) molecule of the LPS. Cultures were grown overnight, washed in 1X PBS, and 50µL of suspension in 1X PBS was mixed with 50µL of 32mM sodium periodate. After 25 minutes of incubation at room temperature, 50µL of 136mM purpald in 2N NaOH was added to each well of a 96 well plate. This was incubated at room temperature for 20 minutes and 50µL of 64mM sodium periodate was added. After 20 minutes of incubation at room temperature, air bubbles were removed using 10µL isopropanol. Samples were read at 540nm using a Biotek Gen5 plate reader. A standard curve was generated using purified *K. pneumoniae* LPS. To ensure quantification of LPS from the same number of bacteria, CFUs/mL were determined and strains were normalized to 10^8 CFUs/mL.

Isolated outer membrane samples were used to visualize the LPS with a 12% SDS-PAGE gel using approximately 4µg of LPS as determined by the purpald assay. Polysaccharide was stained using the ProQ Emerald 300 staining kit by Molecular Probes[79]. High molecular weight polysaccharide indicates smooth LPS, while low molecular weight polysaccharide indicates rough LPS.

Transcriptional expression of outer membrane proteins by qPCR

RNA was extracted from cultures in the exponential phase using the RNeasy kit by Qiagen with DNase treatment. Complimentary DNA (cDNA) was reverse transcribed from 1µg of total RNA using random hexamer primer and the ProtoscriptII reverse transcriptase kit (New England Biolabs). Quantitative polymerase chain reaction (qPCR) was performed using LuminoCt SYBR green on an Eppendorf Mastercycler Realplex 2. Data was analyzed using the ΔΔCT method[81]. Gene expression was normalized to that of *gapA* and clinical isolates were normalized to the lab strain 43816. Primers used are listed in **Table 1.**

Table 1. Primers used for qPCR.

Gene	Forward primer	Reverse primer
gapA	TTGACCTGACCGTTCGTCTGGAAA	AGCATCGAACACGGAAGTGCAAAC
OmpK35	TTCGACAACGCTATCGCACTGTCT	AGTACATGACGGCCGCATAGATGT
OmpK36	CCGTCAACCAGACCGAAGAA	CAGGCCTGAAATTTGGCGAC
ompK26	GAACAACGCCCGGCAAGATGATGA	AGCTGCGGGCATAGACATAGTTCA
lamB	GCGGGTAAACGCTTCTATCA	GGTCAACGTTTTCCAGACCT
ompA	ACGTGCTCAGTCCGTTGTTGACTA	AGTAACCGGGTTGGATTCACCCAT
Lpp	CGGTAATCCTGGGTTCTACTCT	TGCTCAGCTGGTCAACTTTAG

Phagocytosis assay

Murine macrophage cell line RAW 264.7[82] was maintained in 1640 RPMI media supplemented with 10% fetal bovine sera and penicillin, streptomycin, and amphotericin B at 37°C in 5% CO_2. Macrophages were seeded into a 24 well tissue culture plate at $7.5x10^5$ cells/mL and incubated overnight. Overnight bacterial cultures were suspended in a 1X PBS solution to contain approximately $1x10^9$ CFUs/mL. Macrophages were treated with bacteria at a multiplicity of infection (MOI) of 50:1, infection was synchronized using centrifugation at 200xg for 5 minutes, and incubated at 37°C for 15 minutes. Macrophages were washed with 1X PBS to remove non-macrophage associated bacteria and then lysed with 0.1% Triton X-100 in 1X PBS. Solution was

serially diluted, plated on LB agar plates, and incubated overnight at 37°C. To determine the number of macrophage-associated bacteria, colony forming units were counted and CFUs/mL were calculated. Percent phagocytosis was determined by dividing the number of CFUs/mL recovered by the CFUs/mL of the original inoculum.

Statistical Analysis

All experiments were performed with n≥3. Statistical significance was determined using a one-way ANOVA and Tukey's post hoc test using XLSTAT software. Significance compared to 10S+16K is determined at p values of <0.01 (*) and p<0.0001 (**).

Results

Rate of growth, colony morphology, and cell density of *Klebsiella pneumoniae* clonally related clinical isolates

Porins are partially responsible for nutrient acquisition required for the growth and division of the cell. Therefore, porin loss could potentially impact bacterial cultures at all stages of growth. To determine this, standard bacterial growth curves were performed. However, growth curves determined there was no significant difference in growth during the lag, exponential, or stationary phases between any of the strains. (**Figure 5A**). However, clinical isolate 10R+16K did demonstrate a slightly depressed cell density during the stationary phase at 6 and 6.5 hours when compared to 10S+16K (p=.015 and p=.027 respectively). Isolate 10R+16K also exhibited alterations to colony morphology on LB agar streak plating. Colonies were visibly smaller and less mucoid in appearance than those of the other isolates and the lab strain (**Figure 5C**). To determine whether this depressed growth and colony morphology affected cell concentration of an overnight culture, CFUs/mL were found. The 10R+16K strain had similar CFUs/mL as all other strains

indicating that loss of OmpK36 in this strain did not affect bacterial cell concentration of overnight cultures (**Figure 5B**).

Minimum inhibitory concentration is increased with loss of one or both porins in an ESBL background

The clinical isolates showed minimum inhibitory concentrations above the CLSI defined clinical resistance level for both cephalothin (32μg/mL) and gentamicin (8μg/mL) (**Table 2**). The lab strain, with no known antibiotic resistance mechanisms associated with it, was below that threshold for all tested antibiotics. This was predicted as the clinical isolates have previously been characterized as ESBL positive and are expected to be clinically resistant[5]. However, porin loss within the clinical isolates did show an increase in minimum inhibitory concentrations for cephalothin and gentamicin. 10S+16K had an MIC of 125μg/mL for cephalothin and 62.5μg/mL for gentamicin. Loss of OmpK36 resulted in a 2-fold increase in MIC for both cephalothin and gentamicin compared to 10S+16K. Loss of OmpK35 increased the MIC for cephalothin by at least 4-fold compared to 10S+16K but did not alter resistance to gentamicin. Loss of both porins caused a greater than 4-fold increase in the MIC of cephalothin and a 2-fold increase for gentamicin compared to 10S+16K. All clinical isolates and the lab strain were susceptible to colistin.

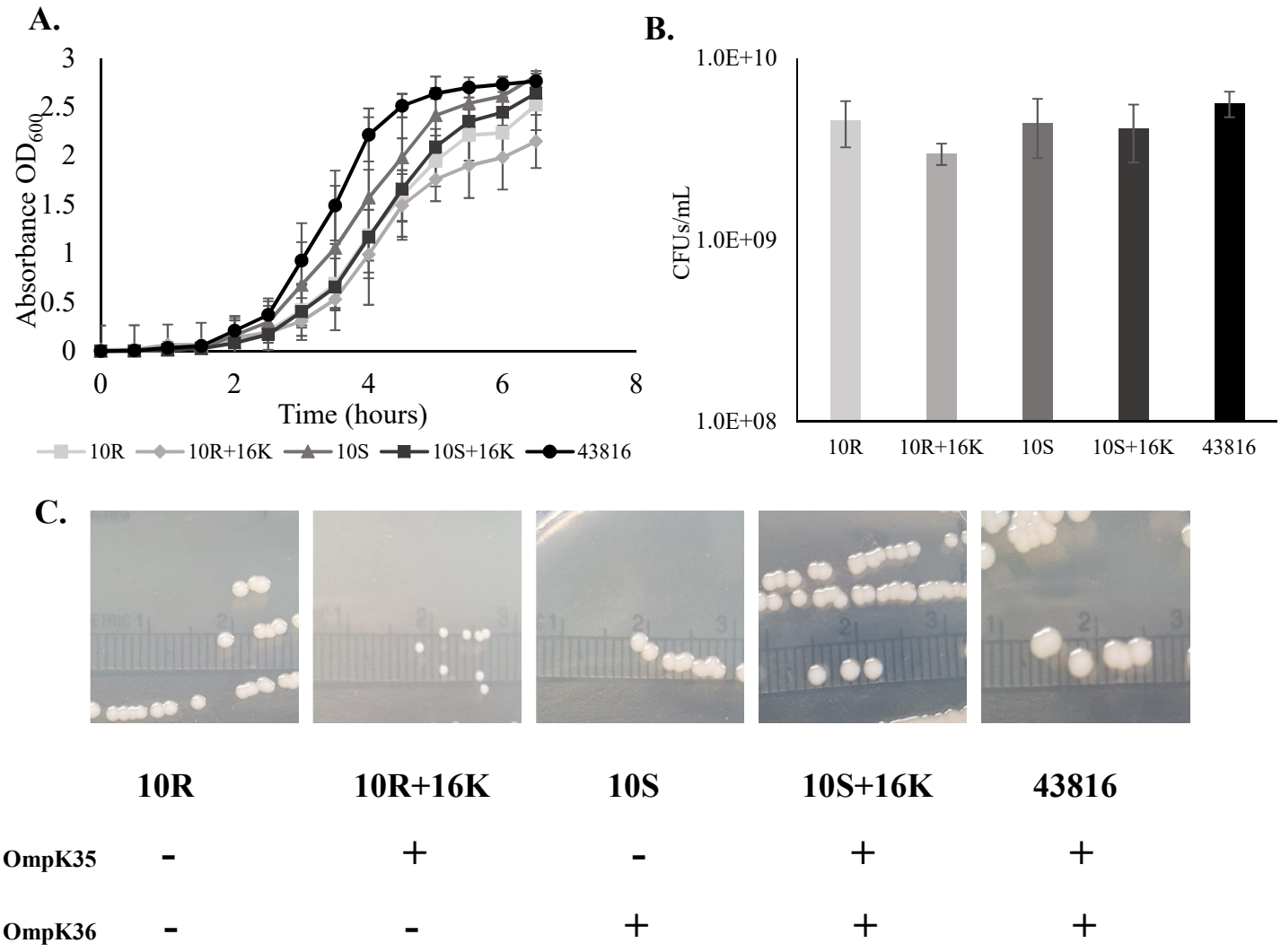


Figure 5. Loss of OmpK36 depresses the stationary phase and alters colony morphology, but does not significantly impact cell density of an overnight culture. A) Standard growth curves of clonally related clinical isolates and a lab strain of *Klebsiella pneumoniae*. OD_{600} was measured every 30 minutes for 6.5 hours. No significant difference among the strains was found using an ANOVA. n=3. Error bars indicate one standard deviation from the mean. B) Overnight cultures were serially diluted, plated onto LB agar plates, and grown overnight at 37°C. Colony forming units were counted and CFUs/mL calculated. Porin loss does not affect CFUs/mL of an overnight culture. n≥5. Error bars indicate one standard deviation from the mean. C) Overnight cultures were streak plated on LB agar plates and grown overnight at 37°C. 10R+16K has visibly smaller and less mucoid colonies than 10S+16K.

Table 2. Minimum inhibitory concentrations were determined using a standard microdilution method. Breakpoint MIC is defined as the concentration at which growth is first inhibitted by at least 90%. Resistance is determined as suggested by the Clinical and Laboratory Standards Institute[29]. Loss of both porins resulted in a 4 fold increase in MIC for cephalothin and a 2 fold increase for gentamicin.

Breakpoint MIC				Porin Expression	
Strain	Cephalothin	Gentamicin	Colistin	OmpK35	OmpK36
10R	>500	125	2	-	-
10R+16K	>500	62.5	1	+	-
10S	250	125	2	-	+
10S+16K	125	62.5	<.5	+	+
43816	2	4	1	+	+
CLSI Resistance	>32	>8	>2		

Capsule production is affected by loss of one or both porins

Capsular polysaccharide was extracted and the production of capsule was indirectly quantified by glucuronic acid concentration (**Figure 6A**). Both 10R +16K and 10S produced significantly less capsule than 10S+16K. Interestingly, loss of both porins in clinical isolate 10R had a contrasting effect on capsule production and had a significant increase. The lab strain 43816 produced similar amounts of capsule as the clinical isolate 10S+16K.

Capsule was also visualized on a 12% SDS-PAGE gel and stained with ProQ Emerald 300 for polysaccharide (**Figure 6B**). There did not appear to be any major differences between isolates, other than a slight decrease in staining for lower molecular weight polysaccharides for 10R+16K. Further investigation into the molecular makeup of the capsule would need to be done to determine whether this absence of staining is due to a decrease in glucuronic acid or another monosaccharide.

Negative staining using 10% nigrosin was used to physically observe the size of the capsule surrounding the bacteria. Visualization at 100X under oil immersion revealed a less distinct capsule for 10R+16K compared to the other isolates (**Figure 6C**).

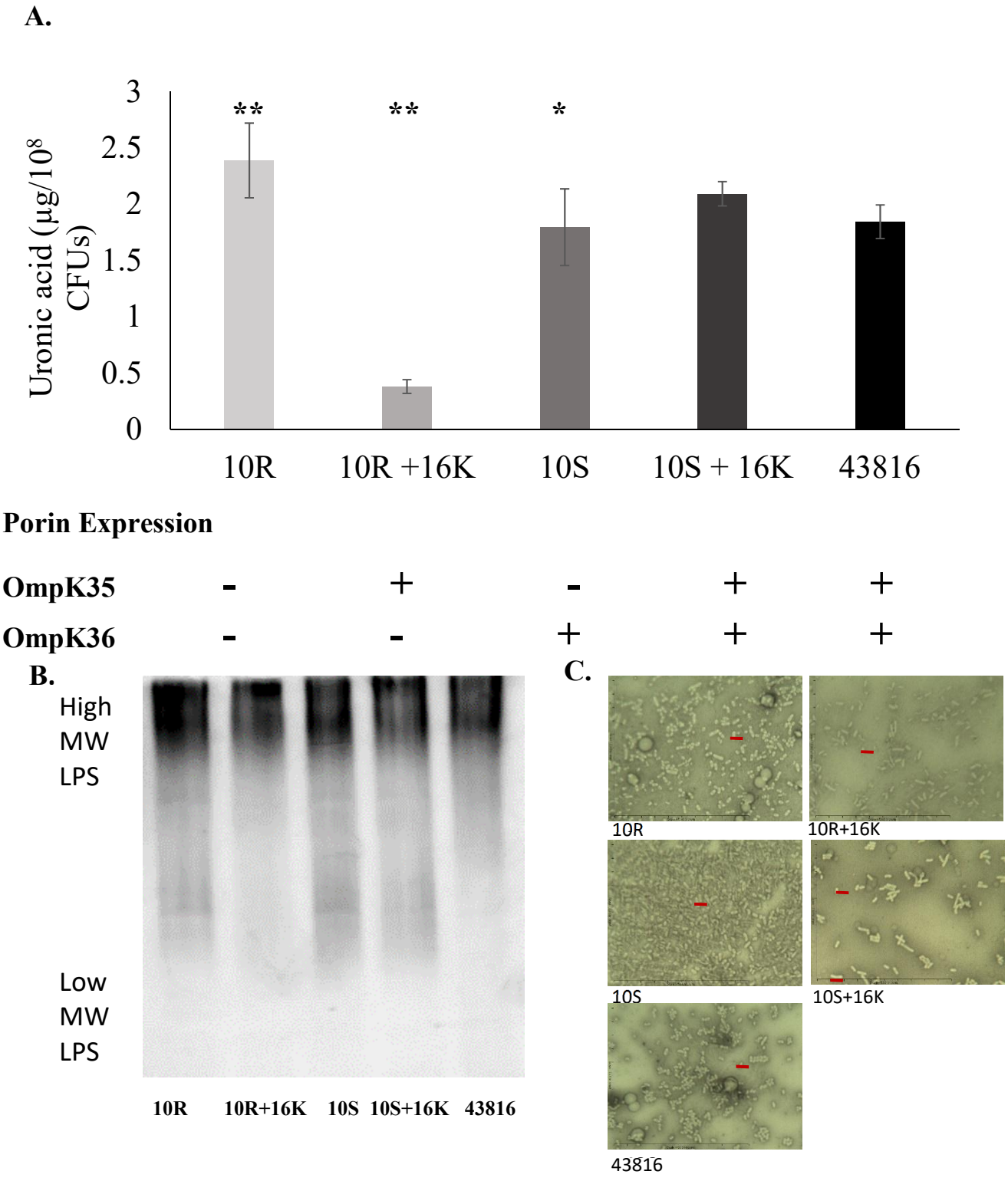


Figure 6. Quantification and visualization of capsular polysaccharide**.** A) Capsule was extracted and glucuronic acid was quantified (µg/10^8 CFUs) using a standard curve. Loss of OmpK35 and OmpK36 alone resulted in significantly decreased capsule production while loss of both porins resulted in significantly increased capsule production. *$p<0.01$, **$p<0.0001$ compared to 10S+16K $n\geq9$. B) Extracted capsule was ran on a 12% SDS-PAGE gel and stained using ProQ Emerald stain. 10R+16K shows a decrease in staining for lower molecular weight polysaccharides compared to 10S+16K. C) Overnight cultures were stained using 10% nigrosin to visually observe capsule at 1000X total magnification. Red line equals 0.58µm.

Lipopolysaccharide content is increased with porin loss

Bacteria were assayed for LPS using the Purpald assay to determine whether porin loss impacted the LPS content (**Figure 7A**). There was a significant difference in the amount of LPS between clinical isolate 10S+16K and the lab strain 43816. Compared to 10S+16K, the isolates with loss of one or both porins exhibited increased LPS content.

To address whether LPS composition was affected by porin loss, purified outer membrane samples were analyzed by SDS-PAGE and stained for polysaccharides using ProQ Emerald stain (**Figure 7B)**. The clinical isolates all exhibited lower molecular weight polysaccharide LPS that is synonymous with less virulent strains, however, the lab strain had higher molecular weight LPS that is indicative of a more virulent strain. This indicates potential differences in LPS structure and composition between the clinical isolates and the lab strain. Clinical isolate 10R+16K did have visibly decreased staining for LPS than all other strains and may have modifications to the LPS due to porin loss.

Transcriptional expression of outer membrane proteins in response to porin loss

Quantitative PCR showed that loss of one or both porins can have a significant impact on the transcriptional expression of other outer membrane proteins. Genes assayed were chosen due to individual importance in compensation for porin function (*ompK26* and *lamB*) or cell envelope stability (*ompA* and *lpp*). Transcriptional expression of *ompK35* and *ompK36* were also assayed and were consistent with porin expression seen in SDS-PAGE gels (**Figure 4B and 4C**). Clinical isolates 10S, 10R+16K and 10R all showed a significant increase in expression of *ompK26* compared to 10S+16K (**Figure 8A**), however only 10R+16K showed a significant increase in expression of *lamB* (**Figure 8B**).

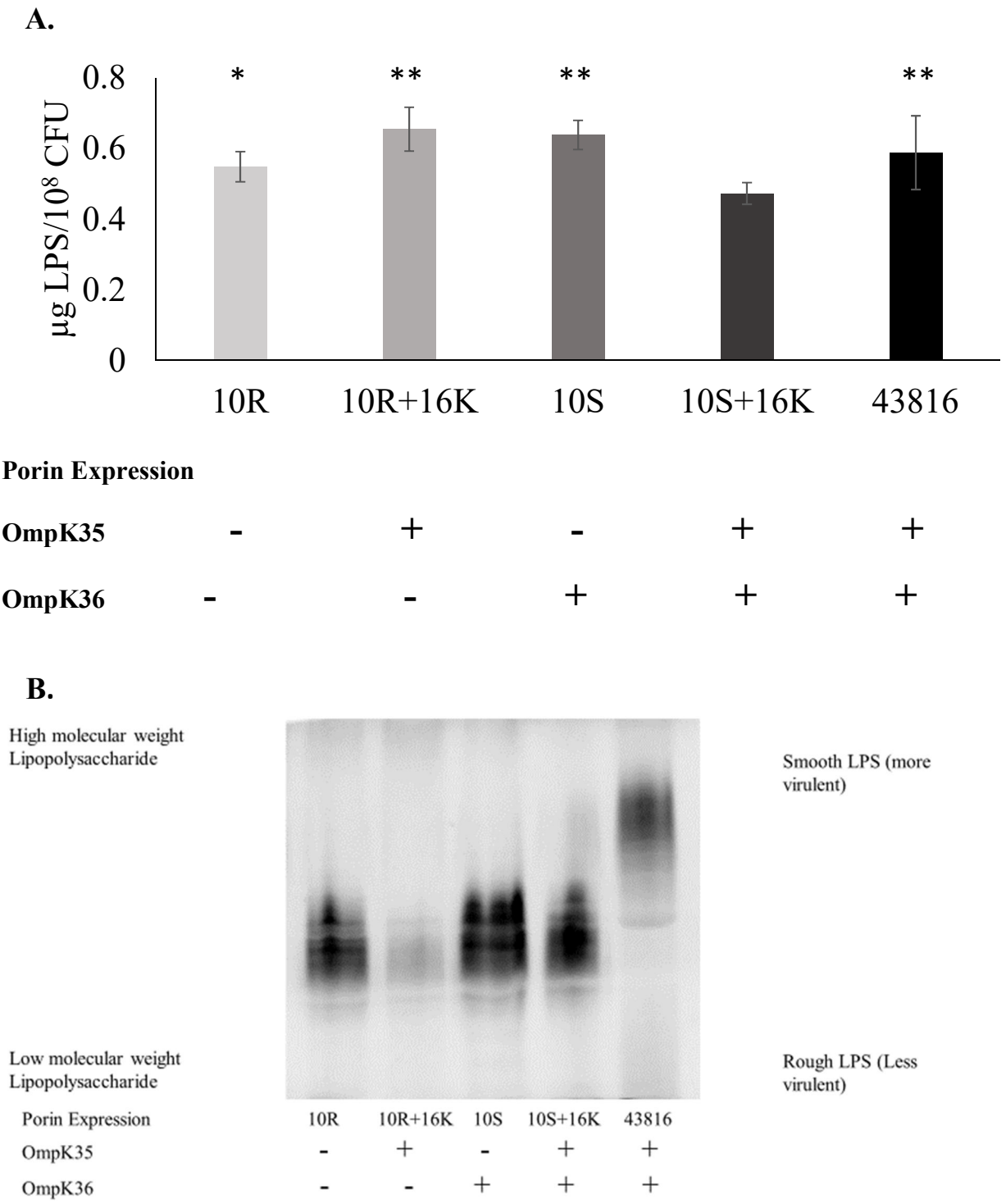


Figure 7. Quantification and visualization of lipopolysaccharide. A) Whole bacteria were assayed for LPS by quantifying the KDO molecule of the LPS through the purpald assay. LPS was measured in µg LPS/10^8 CFUs. *p<0.01, **p<0.0001 compared to 10S+16K. n= 18 from 3 independent cultures. B) LPS composition from isolated outer membrane samples was observed by staining for polysaccharide using ProQ Emerald stain on a 12% SDS-PAGE gel. Clinical isolates exhibit rough LPS synonymous with less virulent form of LPS and the lab strain exhibits smooth LPS more indicative of virulent LPS.

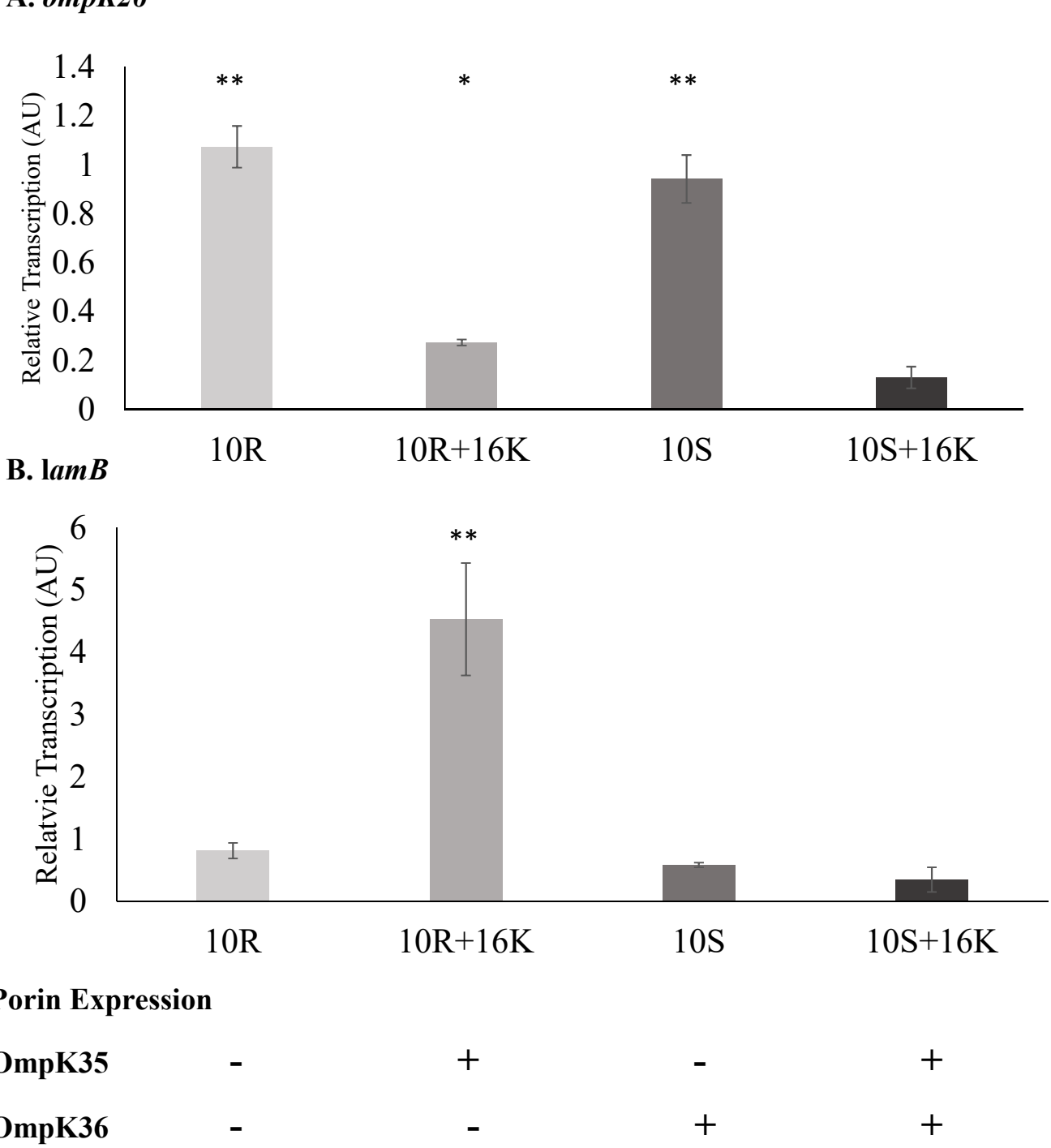


Figure 8. Transcriptional expression of alternative outer membrane proteins by qPCR and analyzed using the ΔΔCt. Genes are normalized to *gapA* and clinical isolates are normalized to the lab strain 43816. A) *ompK26* expression is increased for loss of one or both porins. B) LamB transcriptional expression is increased for loss of OmpK36 only. *p<0.01 **p<0.0001compared to 10S+16K n≥7 from at least 2 independent cultures.

This indicates that loss of OmpK35 or both porins is compensated for by OmpK26, but loss of OmpK36 alone is primarily compensated for by the maltoporin LamB and supplemented with OmpK26.

Both OmpA and Lpp have been previously characterized as being crucial for the maintenance of outer membrane and cell envelope integrity, but have not been thoroughly investigated for their roles in porin loss. The transcriptional expression of *ompA* was decreased for both 10S and 10R+16K when compared to 10S+16K but no significant difference was found for 10R (**Figure 9A)**. Interestingly, expression of *lpp* was significantly increased for 10R+16K, but significantly decreased for 10S and 10R compared to 10S+16K (**Figure 9B**).

Phagocytosis is affected by loss of a single porin

Phagocytosis assays were performed to determine how porin loss alters resistance to phagocytosis by murine macrophages. First, the number of macrophage associated bacteria were determined by counting CFUs recovered from each well. The percent phagocytosis was calculated by dividing the number of recovered bacteria by the number of bacteria in the original inoculum (**Figure 10**). The clinical isolate 10S+16K and the lab strain 43816 showed similar resistance to phagocytosis as there was no significant difference in percent phagocytosis between them. However, 10R+16K and 10S had increased phagocytosis by macrophages compared to 10S+16K. None of the clinical isolates were significantly more resistant to phagocytosis than 10S+16K.

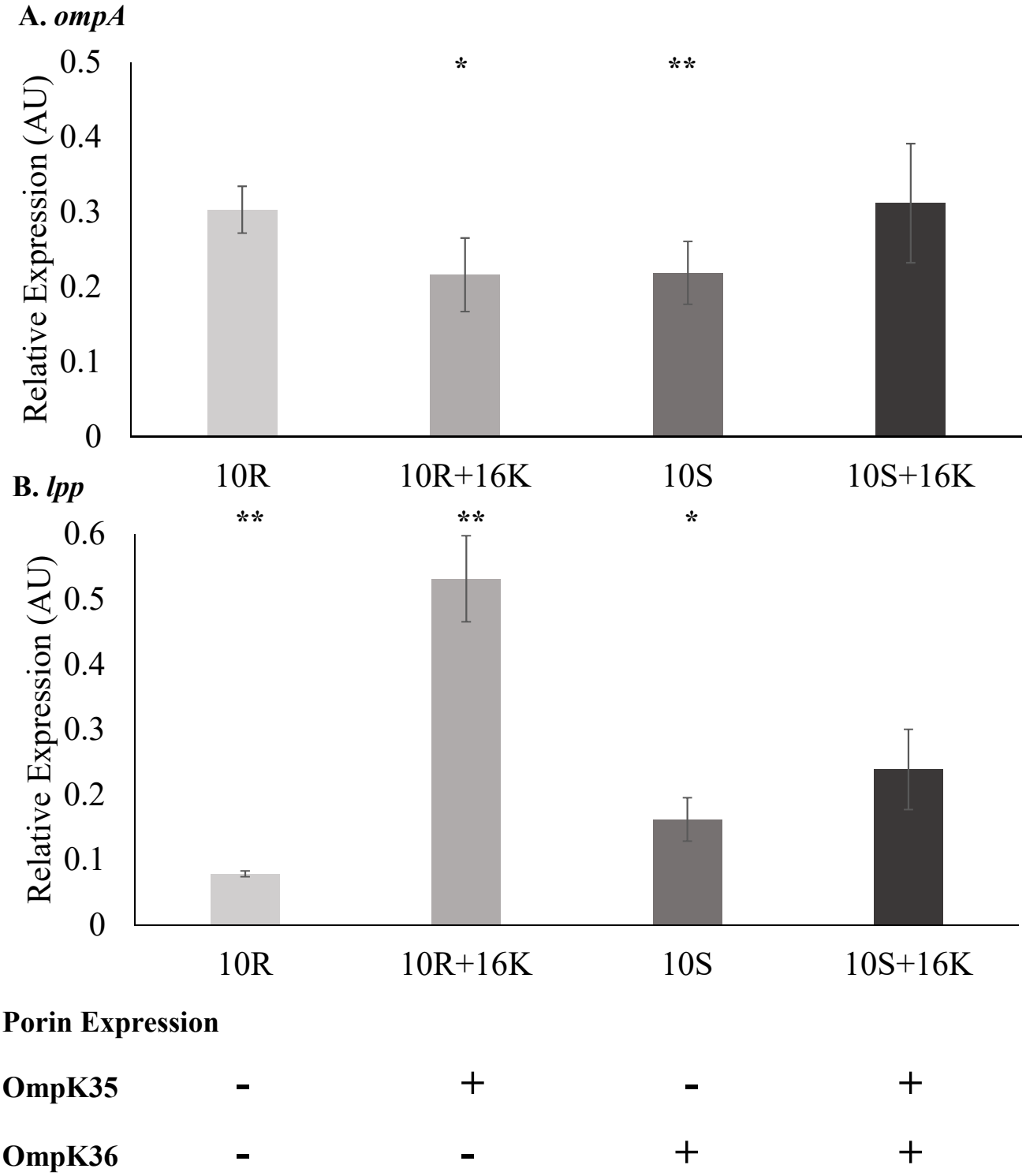


Figure 9. Transcriptional expression of structural outer membrane proteins using qPCR and analyzed using the ΔΔCt. Genes within each isolate were normalized to *gapA* and isolates were normalized to 43816. A) Loss of a single porin resulted in a decrease in expression of *ompA*. B) Loss of OmpK36 resulted in a significant increase in *lpp* expression, but loss of OmpK35 or both porins resulted in a significant decrease. n≥7 from at least 2 independent cultures. *p<0.01 **p<0.0001 compared to 10S+16K.

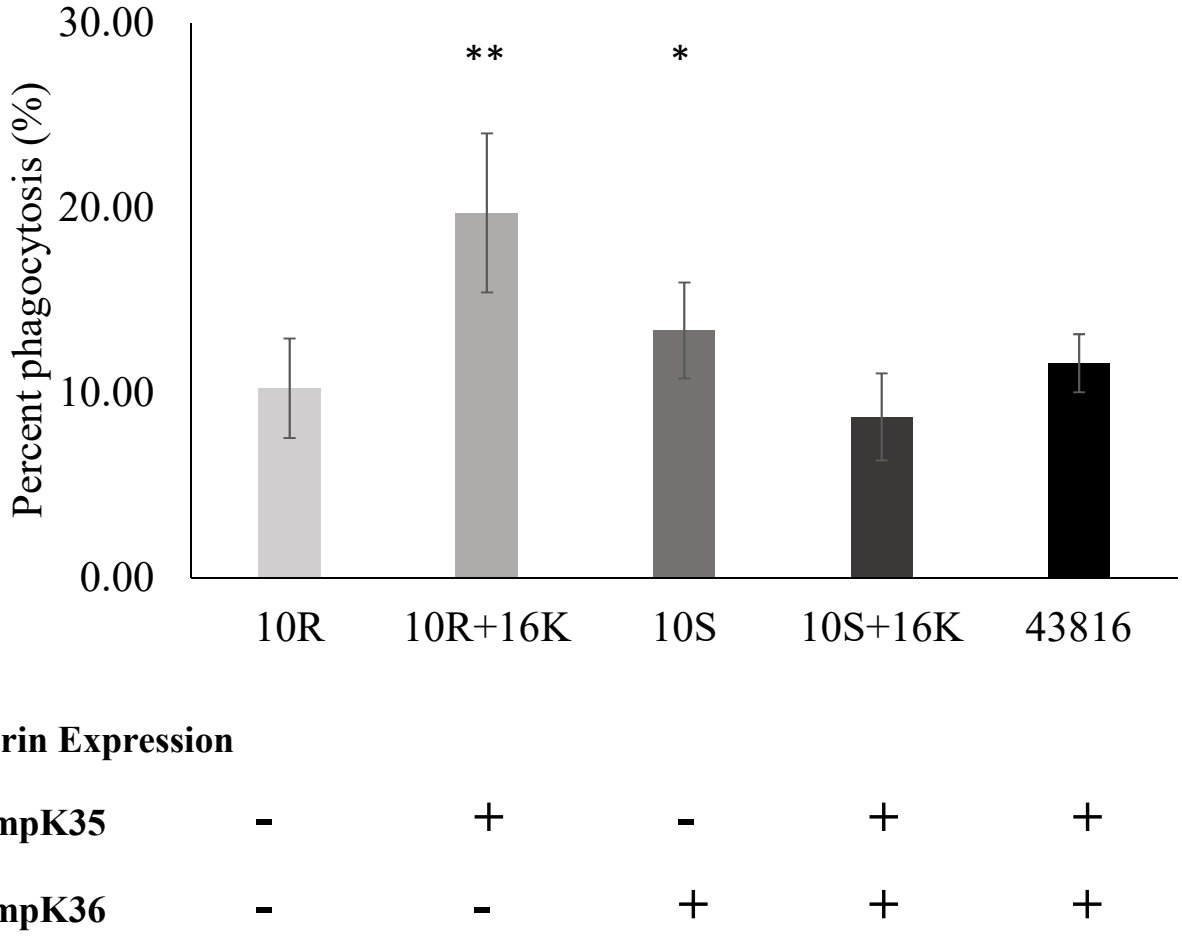


Figure 10. Phagocytosis assays were performed using an MOI of 50:1. Colony forming units from macrophage associated bacteria were counted and CFUs/mL determined. Percent phagocytosis was found by dividing the number of recovered CFUs/mL by the number of CFUs/mL of the original inoculum. The percentage of phagocytosis was increased by loss of OmpK35 ($p<0.0001$) and OmpK36 ($p=0.012$) but not both porins ($p=0.776$) compared to 10S+16K, n=9.

Discussion

This study hypothesized that the loss of OmpK35 and/or OmpK36 would alter the cell surface and result in altered resistance to phagocytosis by macrophages. These isolates adapted to environmental pressures of antibiotics that resulted in loss of one or both porins. Previous studies have focused on the role porin loss plays in antimicrobial and phagocytic resistance, but there is insufficient research looking at the effect porin loss has on other aspects of the cell. Here, it is demonstrated that porin loss has a significant impact on capsule and LPS content, and affects the transcriptional expression of other outer membrane porins. In addition, these cumulative changes alter phagocytic resistance by murine macrophages and may, therefore, impact other aspects of the immune response.

Growth, cell density, and colony morphology were determined to first assess the clinical isolates. While there was a slightly lower OD_{600} in the stationary phase and visibly smaller colonies for loss of OmpK36 (10R+16K), CFUs/mL of an overnight culture (within the stationary phase) were no different than the strain with both porins. This indicates something else is contributing to the differences in colony size and absorbance/cell density for this strain. Capsule production within this strain is also severely decreased compared to 10S+16K (expresses both porins) so it is possible that these differences are due to a decrease in the capsular polysaccharide surrounding the bacteria.

Glucuronic acid quantification and negative staining showed that the amount of capsule produced by each strain was significantly decreased by loss of either porin (10R+16K and 10S), but significantly increased by loss of both (10R). Previous studies of the relationship between porin loss and capsule production are contradictory. One study showed that capsule production decreased in an *ompK36* knockout[25] and another described an *ompK36* knockout as being more mucoid in appearance, indicating an increase in capsule production[10]. Interestingly, these

contrasting studies used *K. pneumoniae* strains with different capsular serotypes (K1 and K2 respectively) than the strains tested here (unknown). Capsule serotype is based on the specific structure of the capsular polysaccharide for each strain. This structure is antigenic and can be recognized by specific antibodies[50,51,57]. It is therefore possible that the impact porin loss has on the production of capsule could be dependent on serotype.

The serotypes of the strains used in this study were unable to be determined by PCR using previously published primers for K1, K2, and K5 serotypes (data not shown)[51]. Further analysis of the capsule using antigenic serotyping[51] would need to be performed to determine the specific serotype of these isolates. However, it does appear that the effect of porin loss in these clinical isolates is similar to those of the K1 serotype seen by Srinivasan *et al.*[25]. The direct relationship between capsule and porins has yet to be investigated. It is unknown whether porins are responsible for the uptake of the specific monomeric sugars responsible for the creation of the polysaccharide of the K-antigen. Therefore, it is possible that porin loss could directly impact the ability of the capsule to be synthesized. This could be tested by treating strains of different serotypes that exhibit porin loss with radiolabeled sugars. The capsule could be extracted and the amount of radiolabeled sugars within the capsule determined. If porin loss affects the import of sugars used in capsule synthesis, then these strains will contain less radiolabeled capsule. However, data collected in this investigation does not seem to support this as loss of a single porin decreased capsule and loss of both porins increased the amount of capsule. For this hypothesis to have been supported, loss of both porins would need to have further decreased capsule production.

Capsular polysaccharide has been identified as a primary indicator for low levels of phagocytosis. The presence and serotype of the capsule decreases the ability of the phagocytic cell to recognize, attach, and internalize bacteria. Non-capsule producing *K. pneumoniae* are

phagocytosed at higher rates than strains that produce a capsule[83,84]. The capsular serotype can also have a dramatic effect on strain virulence as well. K1 and K2 serotypes are notably more virulent and resistant to phagocytosis than other serotypes[50,51,78,84]. The clinical isolates used in this study were unable to be serotyped based on PCR methods and are likely not of the K1, K2, or K5 serotype. The lab strain 43816 was previously characterized as K2 serotype[57]. The phagocytosis assays performed here showed no difference between the lab strain and the clinical isolate expressing both porins (10S+16K). The capsular polysaccharide assays from this study also showed no significant difference between the clinical isolate expressing both porins and 43816. This indicates the capsular serotype of the clinical isolates confers equal protection to phagocytosis as the K2 serotype.

It was hypothesized that porin loss related capsule production would correlate with resistance to phagocytosis. This was partially supported as loss of a single porin decreased the amount of capsule produced by the clinical isolates and increased the percent phagocytosis. In contrast, the strain 10R that expressed neither porin produced significantly more capsule, but was not more resistant to phagocytosis than the isolate that expressed both porins (10S+16K). If capsule was the primary factor for the resistance to phagocytosis, then the increase in capsule for 10R would have been expected to decrease the percent phagocytosis. However, this was not the case, indicating that other factors were impacting phagocytosis. This is most likely porin expression as 10R expresses neither porin. In previous studies using *K. pneumoniae* clinical isolate NVT2001, loss of both porins led to an increase in phagocytosis by human neutrophils *in vitro*[10]. Therefore, it is possible that in this study the increase in capsule produced by 10R provided some protection against phagocytosis even with the dual porin loss background. However, the capsule was not sufficient to further decrease percent phagocytosis.

Capsule is not the only polysaccharide-based molecule altered by porin loss. The data presented here demonstrate that loss of one or both porins also significantly increases the LPS content in cells. Bacteria have a preferred surface area to volume ratio that is maintained for optimum acquisition and distribution of nutrients throughout the cell[85]. OmpK35 and OmpK36 are two of the most abundant proteins of the outer membrane. Therefore, loss of one or both porins would likely result in a decrease in the surface area of the bacteria. The increase in LPS content in the outer membrane could be a compensatory mechanism for maintaining a preferred surface area to volume ratio in response to porin loss.

The purpald assay quantifies the content of the LPS by measuring the KDO molecule, but does not provide any information regarding the composition of the core antigen or the O antigen of the LPS. Therefore, the LPS was also analyzed using SDS-PAGE to test for polysaccharide. There was a visible difference in the molecular weight of the LPS between the clinical isolates and the lab strain 43816 that is likely due to genetic differences between the lab strain and the clinical isolates. The clinical isolates exhibited a lower molecular weight polysaccharide that is indicative of rough LPS, while the lab strain exhibited higher molecular weight indicative of smooth LPS. Rough LPS has modified O-antigen or has completely lost the O-antigen. Loss of the O-antigen has been shown to be irreversible in some strains and disables antigenic switching. This decreases the ability of the bacteria to change its surface structure as a way to evade the immune system[86]. Rough LPS has also been described to be more sensitive to complement-mediated killing. This is because the C3-b complement protein will bind closer to the membrane, which allows for the formation of the membrane attack complex and the cell is lysed[87]. Smooth LPS positions the C3-b complement protein farther away from the outer membrane and the membrane attack complex is not formed[58].

Staining for polysaccharide from an outer membrane sample of showed a visible decrease for the loss of OmpK36 in strain 10R+16K compared to 10S+16K (expresses both porins). The gel was loaded with equal amounts of LPS as per the purpald assay, therefore a decrease in staining for polysaccharide indicates alterations to the core antigen or O antigen segments. Lipopolysaccharide has been well documented as a virulence factor for *K. pneumoniae*[2,3,58], and the presence of LPS has been shown to aid in evasion of phagocytosis as well. LPS core polysaccharide core mutants were shown to be more susceptible to phagocytosis than wildtype, however, O-antigen mutants were not[2]. In this study, loss of one or both porins (10R+16K, 10S, and 10R respectively) resulted in a significant increase in the amount of LPS of each strain, and loss of OmpK36 (10R+16K) resulted in decreased polysaccharide staining. These trends do not correlate with those seen for resistance to phagocytosis here, so it is unlikely that alterations to the LPS due to porin loss altered the resistance to phagocytosis. However, alterations to the O-antigen or core antigens could potentially impact other interactions with the immune system. Specifically, activation of the classic complement cascade and toll-like receptor 4 (TLR-4) which would increase secretion of pro-inflammatory cytokines by the macrophages. Activation of TLR-4 would likely increase for 10R+16K (loss of OmpK36) if alterations to the O-antigen or core antigens made the pathogen associated molecular pattern for the lipid A portion of the LPS more accessible[88]. Further inquiry into the makeup of the O-antigen of these strains using mass spectrometry would be helpful to determine where the molecular differences lie between the LPS of the porin loss isolates. This would also give more evidence as to whether alterations to the LPS due to porin loss contributed to the decrease in resistance to phagocytosis observed by loss of OmpK36 (10R+16K).

The transcriptional expression of other outer membrane proteins was also significantly different between isolates exhibiting porin loss (10R+16K, 10S, and 10R) and the isolate expressing both porins (10S+16K). This study demonstrated that loss of one or both porins was compensated for by the upregulation of other specific porins. Transcriptional expression of *ompK26*, which is specific for oligo galacturonate (sialic acid) and is impermeable to large β-lactam antibiotics, was significantly increased for isolates with loss of OmpK35 (10S), OmpK36 (10R+16K), or both porins (10R). However, loss of OmpK36 (10R+16K) did not result in upregulation of *ompK26* to the same degree as loss of OmpK35 (10S) or both porins (10R) did. Previously, OmpK26 was shown to be essential for compensation of OmpK36. The Alberti lab attempted to create an *ompK26* knockout in the strain 10R (expresses neither porin) used in this study, but were unsuccessful without the reversion of OmpK36. This demonstrates that OmpK26 may indispensable when both OmpK35 and OmpK36 are lost[40]. Therefore, the significant upregulation of *ompK26* seen here was to be expected.

Alberti *et al.* 2011 also showed that expression of OmpK26 without expression of OmpK36 reduced the virulence of *K. pneumoniae.* Experiments testing the lethality in a mouse intraperitoneal infection model revealed the strain that expressed OmpK36 but not OmpK26 was significantly more fatal. This indicates that OmpK36 is significantly more important for survival of the bacteria in a mouse model than OmpK26 is. In the data presented here, the isolates exhibiting loss of either one or both porins also had increased transcriptional expression of *ompK26,* however, the expression of OmpK26 was not indicative of evasion of phagocytosis. It would be expected that strains with upregulation of *ompK26* would also have the highest percent phagocytosis. However, the strains with the highest expression of *ompK26,* 10R (expresses neither porin) and

10S (loss of OmpK35), were not the most susceptible to phagocytosis. Therefore, it is unlikely that *ompK26* expression is a good indicator for the resistance to phagocytosis.

LamB is a porin that primarily imports the sugar maltose, although other sugars can also diffuse through this channel. The strain expressing only OmpK35 (10R+16K) had a significant increase in *lamB* expression. Previous experiments have shown a *lamB* knockout in an *ompK35/ompK36* negative background triggers the expression of *ompK26* and increases the resistance to meropenem (a carbapenem antibiotic)[41]. It was therefore concluded that LamB may compensate for the loss of OmpK36. The current study supports this hypothesis (**Table 3**). The clinical isolate 10R+16K (loss of OmpK36), expressed *ompK26* to a lesser degree than 10R (loss of both porins) and 10S (loss of OmpK35) and also expressed *lamB* more than 10R and 10S. This study provides evidence that OmpK35 and OmpK36 each have individual compensatory porins, OmpK26 and LamB respectively.

Table 3. Transcriptional expression of porins in clinical isolates.

Strain	*ompK35*	*ompK36*	*ompK26*	*lamB*
10R	-	-	++	-
10R+16K	+	-	+	++
10S	-	+	++	-
10S+16K	+	+	-	-

LamB has not been previously characterized as a virulence factor. Interestingly, the strain expressing the highest relative transcription of *lamB*, 10R+16K (loss of OmpK36), was also the strain that had the greatest susceptibility to phagocytosis. Further inquiry into the role LamB plays in pathogenicity should be done. The specific focus should be to determine whether loss of OmpK36 or increased LamB on the cell surface increases susceptibility to phagocytosis.

The structural outer membrane protein, OmpA, is a highly-characterized protein that has been shown to be multifunctional. This protein has some porin functionality[89], is important for the stability of the cell envelope[27], acts as an adhesin to attach the cell to surfaces[43], and acquires iron within the host[46]. Turner *et al.* 2016 noted that OmpA content was enriched for outer membrane vesicles from isolates with loss of OmpK35 (10S), but not for loss of OmpK36 (10R+16K) or both (10S+16K). This study was performed using the same clinical isolates from the present study[47]. Outer membrane vesicles are derived from the outer membrane and can be representative of the outer membrane[4]. The data from this study contrasts this finding, as loss of either porin (10R+16K or 10S) showed a significant decrease in *ompA* transcription, which is not necessarily indicative of protein content. However, previous literature has confirmed selective sorting mechanisms for proteins into outer membrane vesicles and a distinct difference between protein content of the outer membrane and outer membrane vesicles[3,4]. Therefore, it is possible that although transcription of *ompA* decreases due to loss of a single porin, OmpA is still enriched in outer membrane vesicles due to this selective sorting mechanism.

OmpA is perhaps the most studied outer membrane protein of Gram-negative species. It has been well documented for its role in virulence. March *et al.* showed that *K. pneumoniae ompA* knockouts were susceptible to phagocytosis to the same degree as *ompK36* knockouts[2]. Previous work from the same lab also showed that OmpA deficient bacteria were more likely to induce an inflammatory response necessary to clear an infection than the strain that expressed OmpA both *in vitro* by A549 lung epithelial cells and *in vivo* in a mouse model[45]. Together these results indicate that OmpA contributes to the evasion of the immune response. The current data agree with this finding because the clinical isolates 10R+16K (loss of OmpK36) and 10S (loss of OmpK35), which expressed significantly less *ompA* than 10S+16K (expresses both porins), were

also the most susceptible to phagocytosis. The isolate 10R (loss of both porins), which expressed similar amounts of *ompA* in comparison to 10S+16K (expresses both porins), also showed similar percent phagocytosis. Therefore, the expression of OmpA observed in this study may be a driving factor in whether the bacteria would be more resistant or susceptible to phagocytosis by murine macrophages.

The transcriptional expression of *lpp* showed that loss of OmpK35 (10S) or both porins (10R) resulted in decreased expression of *lpp,* but loss of only OmpK36 (10R+16K) resulted in a significant increase in expression. This indicates that *lpp* expression may be sensitive to OmpK35. While the role of *lpp* has been well documented for cell envelope stability[27], information on Lpp expression under harsh conditions is lacking. This includes the role Lpp plays in response to porin loss, exposure to antibiotics, or other toxic compounds. A more in-depth analysis regarding the impact of porin loss on the expression of proteins such as OmpA and Lpp should be done to determine the overall effect of porin loss on the stability of the cell envelope.

Previous literature indicates that Lpp expression confers protection from phagocytosis by neutrophils *in vitro* and may be important for maintaining cell envelope integrity within the host[48]. However, in the present experiments the clinical isolate with the highest *lpp* expression was also the least resistant to phagocytosis. This isolate, 10R+16K, was also deficient in OmpK36 and capsule, which are both indicators of resistance. It is likely then, that OmpK36 and capsule are better indicators of resistance or susceptibility to phagocytosis than Lpp.

Lastly, this study showed that loss of either porin individually correlated with a significant increase in percent phagocytosis. This data contrasts slightly with previous literature. Tsai *et al.* 2011 created isogenic knockouts of *ompK35, ompK36*, and both porins. Exposure to phagocytic human neutrophils *in vitro* showed that loss of OmpK36 or both porins significantly increased

phagocytosis while loss of only OmpK35 had no effect[10]. A similar study by March *et al.* 2013 showed that loss of OmpK36 resulted in an increase in phagocytosis by murine macrophages *in vitro*, but did not look at the effect of the loss of OmpK35 or that of both porins[2]. Here it was demonstrated that the loss of OmpK35 (10S) or OmpK36 (10R+16K) showed an increase in phagocytosis by murine macrophages, with the loss of OmpK36 having a greater increase in phagocytosis than loss of OmpK35 did. The loss of both porins (10R) did not have any effect on phagocytosis though, which is different from the study done by Tsai *et al* 2011. The differences seen between this and previous studies could lie in the other changes observed in the bacteria due to porin loss or could be due to the differences between the human neutrophils and murine macrophages used in each study.

The clinical isolates with loss of only one porin (10R+16K and 10S) also exhibited a decrease in capsule production and expression of the virulence factor *ompA.* Previous literature indicates that these strains should also have been more susceptible to phagocytosis based on these specific changes[2,50,90,56]. The data on single porin loss from this study supports this. However, the clinical isolate 10R which expressed neither porin should have been more susceptible to phagocytosis based on porin expression, but this isolate also exhibited increased capsule production and equal *ompA* expression to the isolate with both porins (10S+16K). Both factors could have compensated for the loss of both porins and provided some protection from phagocytosis.

The research performed here sheds light on the makeup of the cell envelope in porin loss clinical isolates, as well as the effect porin loss has on virulence. Here, it is demonstrated that capsule production, expression of OmpK35 and OmpK36, as well as transcriptional expression of *ompA* are the most likely indicators for resistance to phagocytosis by macrophages. However, this

study also points out some gaps in knowledge that should be addressed in future research. First and foremost, the role porin loss plays in capsule production across serotypes should be determined. Assays should be specifically designed to determine how porin loss affects the amount of capsule produced and the composition of the capsule amongst different serotypes.

Secondly, this study demonstrated that porin loss could potentially affect the LPS content and composition. The loss of one or both porins significantly increased the content of LPS compared to the isolate expressing both porins (10S+16K). However, loss of OmpK36 (10R+16K) showed a decrease in the staining of polysaccharide, indicating alterations to the composition of the core or O antigen polysaccharides. This especially should be determined as alterations to the O-antigen or core polysaccharides could potentially alter the inflammatory response of the host[88] and alterations to the core polysaccharide could increase susceptibility to phagocytosis[2].

Lastly, the effect that porin loss has on the virulence factor OmpA should be more closely determined. Previous studies done by this lab indicate that loss of one or both porins causes an enrichment of OmpA in OMVs[47], but the current study shows that transcription of *ompA* decreases with the loss of one porin (10R+16K and 10S). A better quantitative measurement of OmpA protein in porin loss species should be constructed to determine what affect porin loss has on the amount of OmpA protein in the outer membrane. This would aid in determining whether OmpK35, OmpK36, or OmpA contribute more to resistance to phagocytosis than the transcriptional expression of *ompA* does.

The aim of this study was to investigate if the cumulative changes due to porin loss influence the resistance to phagocytosis. It was demonstrated that loss of one or both porins alters capsule production, LPS content, and the expression of other outer membrane proteins. These

changes to the cell surface in response to porin loss had a cumulative impact on phagocytosis by macrophages.

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Vita

Debra N Brunson obtained her BS in Microbiology in 2013 from Idaho State University in Pocatello, Idaho. While there she published a paper titled “Antigen Detection in Canine Blastomycosis: Comparison of Different Antibody-Antigen Combinations in Two Competitive ELISAs”. Debra has spent the last 3 years working on her thesis research in pathogenic microbiology under mentor Dr. Terri N Ellis and was part of a publication titled “Porin loss impacts the host inflammatory response to outer membrane vesicles of *Klebsiella pneumoniae*”. She also presented her research at the national meeting for the American Society for Microbiology in May of 2015. After graduation Debra hopes to be accepted into a PhD program to continue research on pathogenic bacteria and immunology.