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Porin loss in *Klebsiella pneumoniae* clinical isolates impacts production of virulence factors and survival within macrophages

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ABSTRACT

Clinical isolates of *Klebsiella pneumoniae* are often resistant to beta-lactam antibiotics via the acquisition of extended spectrum beta lactamase (ESBL) enzymes paired with loss of one or both major outer membrane porins. It has been well established that loss of OmpK35 and/or OmpK36 correlates with increased minimum inhibitory concentrations of antibiotics that target the peptidoglycan. However, little is known concerning the downstream effects porin loss might have on other major virulence factors such as the polysaccharide capsule or LPS. Furthermore, it is unknown whether these cumulative changes impact pathogenesis. Therefore, the focus of this study was to identify alterations in production of the major virulence factors due to porin loss; and to investigate the effect these changes have on host pathogen interactions. Our data demonstrates that loss of a single porin is paired with reductions in capsule, increased LPS content, and up-regulated transcription of compensatory porin genes. In contrast, loss of both porins resulted in a significant increase in capsule production. Loss of OmpK35 alone or dual porin loss was further associated with reduced oxidative burst by macrophages and increased ability of the bacteria to survive phagocytic killing. These data indicate that porin loss is accompanied by a suite of changes in other virulence-associated factors. These cumulative changes act to nullify any negative fitness effect due to lack of the nonspecific porin proteins, allowing the bacteria to grow and survive phagocytic immune responses.

1. Introduction

Porins are trimeric transmembrane beta-barrel proteins found in the outer membrane of Gram-negative bacteria. These proteins create channels of defined size, allowing for nonspecific diffusion of hydrophilic solutes and other small molecules through the outer membrane. Bacteria of the *Enterobacteriaceae* commonly express two major nonspecific porins with similar functions but differing in their permeability to small molecules. While porins play an important role in nutrient acquisition, the nonspecific nature of these porins also allows the passage of antibiotics, such as the β-lactams (Delcour, 2009; Nikaido, 2003).

In *Klebsiella pneumoniae*, the major nonspecific porins are OmpK35 and Ompk36, which are homologues to the *Escherichia coli* OmpF and OmpC, respectively (Alberti et al., 1995; Dutzler et al., 1999; Nikaido, 2003). Clinical isolates of *K. pneumoniae* that exhibit antibiotic resistance via expression of Extended Spectrum β-lactamase (ESBL) or carbapenemase enzymes (KPC) commonly exhibit the loss of either OmpK35 or both OmpK35 and OmpK36 (Ardanuy et al., 1998; Doumith et al., 2009; Kaczmarek et al., 2006). Investigations of antibiotic resistant isolates have demonstrated that decreased outer membrane porin expression is correlated with an increased minimum inhibitory concentration (MIC) to multiple antibiotics, most commonly those that target peptidoglycan synthesis (Hernandez-Alles et al., 1999; Kaczmarek et al., 2006; Pages et al., 2015).

Porin loss in *K. pneumoniae* is a clinically important phenomenon that has been shown to alter the antibiotic resistance profile of the bacteria. However, there is a gap in our understanding of how this alteration of the fundamental architecture of the bacterial envelope impacts other aspects of bacterial physiology and pathogenic mechanisms. In this study, we investigated the impact of specific porin loss on production of the major bacterial virulence factors of *Klebsiella*, utilizing a panel of clonally related clinical isolates with differential expression of OmpK35 and OmpK36 (Ardanuy et al., 1998; Domenech-Sanchez et al., 2003; Turner et al., 2016). Specifically, we investigated the effect of differential porin expression on capsule, lipopolysaccharide, and alternative porin expression. These aspects were chosen as previous investigations have indicated that these components directly contribute to the ability of the bacteria to survive within the human host (Clegg and Murphy, 2016; Paczosa and Mecsas, 2016).

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Our study demonstrates that loss of a single porin is paired with reductions in capsule, increased LPS content, and up-regulated transcription of compensatory porin genes. In contrast, loss of both porins resulted in a significant increase in capsule production. Significantly, when exposed to macrophages, the increased production of capsule and other virulence factors was directly related to the ability of *Klebsiella* to survive phagocytic killing. This is the first study to fully investigate the relationship between antibiotic resistance associated porin loss and their impact on the major virulence factors of *Klebsiella*. Our data demonstrates that changes in gene expression related to resistance and virulence are closely linked and have a direct impact on the relative virulence of the bacteria.

2. Methods

2.1. Bacterial strains, plasmids, and media

Klebsiella pneumoniae isolates were graciously provided by Dr. Sebastian Albertí, University of the Balearic Islands. Strains CSUB10S and CSUB10R are clonally related ESBL positive clinical isolates that have been previously characterized as exhibiting different patterns of expression of the OmpK35 and OmpK36 porins (Ardanuy et al., 1998; Domenech-Sanchez et al., 2003). CSUB10S expresses only OmpK36, while CSUB10R expresses neither OmpK35 nor OmpK36. A plasmid containing the gene, with native promoter, for *ompK35* (pSHA16 K) was transformed into both parental strains to create CSUB10R + pSHA16 K (expresses only OmpK35) and CSUB10S + pSHA16 K (expresses both porins). All cultures were grown at 37 °C with agitation (200 rpm) in Luria Bertani (LB) broth (Difco, Detrot, MI.) with 16 μg/mL cephalothin, and strains with plasmids were grown in cephalothin and 50 μg/mL kanamycin for plasmid maintenance. All antibiotics and chemicals are from Sigma, St. Louis, MO. unless otherwise noted.

The *K. pneumoniae* lab strain ATCC 43816 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 less virulent strain that expressed both porins.

2.2. Outer membrane isolation

The outer membrane was isolated using previously described methods (Cahill et al., 2015). Briefly, 100 ml cultures were grown in LB Broth. with appropriate antibiotics to an OD_{600} between 1.5 − 2.0. Cultures were centrifuged at 10,000 xg for 10 min, the cell pellet resuspended in Tris-sucrose solution (20 mM Tris, 20% sucrose, pH 8.0), and the cell wall was digested using lysozyme (15 mg/mL) in 0.1 M EDTA solution for 40 min. A 0.5 M $MgCl_2$ solution was added to the lysate and centrifuged at 12,000 ×g for 20 min. The pellet was dissolved in 10 mM Tris (pH 8.0), sonicated on ice, and cellular debris was pelleted. The supernatant containing membranes was centrifuged at 40,000 ×g for one hour and resuspended in deionized water. The membrane fraction was treated with Sarkosyl solution at room temperature for 20 min and centrifuged at 40,000xg for 90 min. The pellet containing outer membrane was resuspended in 1X PBS. Protein concentration was determined using a standard Bradford assay (Coomassie Plus, Thermo Fisher Scientific, Waltham, MA.). Proteins were visualized on a 12% SDS-PAGE gel using the SYPRO Ruby stain from Molecular Probes.

2.3. Capsular polysaccharide characterization

Capsular polysaccharide (CPS) was extracted using the protocol outlined by Domenico et al. (Domenico et al., 1989). Five hundred microliters of an overnight culture were mixed with 100 μL of 1% Zwittergent 3–14 in 100 mM citric acid, pH 2.0. The mixture was vortexed vigorously, incubated at 50 °C for 20 min, and centrifuged for 5 min at 14,000 rpm. The supernatant was then transferred to a fresh tube, mixed with 1.2 mL of absolute ethanol, and incubated for 90 min at 4 °C. Precipitate was collected after centrifugation at 14,000 rpm for 10 min and dried at room temperature.

CPS was quantified following a previously established protocol by Lin et al (Lin et al., 2012) that is a modification of the original protocol by Domenico et. al. (Domenico et al., 1989). Purified CPS, solubilized in water, was vortexed vigorously with 1.2 mL of 12.5 mM sodium tetraborate in concentrated sulfuric acid and heated for 5 min at 95 °C. The samples were then cooled before the addition of 20 μL of 0.15% m-hydroxydiphenyl, and measured the absorbance at 540 nm A standard curve was generated using D-glucuronic acid to determine the concentration of glucuronic acid of the CPS samples. To ensure quantification of CPS from the same number of bacteria, strains were normalized to 10^8 CFUs/mL. Each assay was performed in triplicate from six individual cultures.

The sugar composition of the CPS was further analyzed by GC–MS. For this analysis the CPS was purified from LPS using sodium deoxycholate as previously described by Kachlany et al. (Kachlany et al., 2001). Dry CPS was dissolved in 500 μl of DI water containing sodium deoxycholate to final concentration of 6 mM and vortexed until dissolved. The mixture was then incubated at 65 °C for 15 min, and cooled on ice an ice bath in the refrigerator for 15 min. Acetic acid was added to a final concentration of 1.2% and the samples were centrifuged at 20,000 ×*g* for 15 min. After centrifugation, 300 μl of the supernatant was added to a new tube and absolute ethanol was added to a final concentration of 80%. The samples were cooled on ice in the refrigerator at 4 °C for 60 min, centrifuged at 20,000 ×*g* for 15 min, and the ethanol was decanted. The pellet was dissolved in 400 μl of DI H_2O, frozen at −80 °C. and then freeze-dried until later analysis.

Purified CPS was hydrolyzed and prepared for analysis by gas chromatography mass spectrometry (GC/MS) as previously described by York et al. (York et al., 1986). The extracted and purified CPS from the treated and untreated cells was hydrolyzed in 0.5 M HCl at 85 °C for 18 h. After the incubation period, the HCl was evaporated at 40 °C using a gentle stream of air. The dried carbohydrates were dissolved in 500 μl of DI water and evaporation was carried out once again.

Re-*N*-acetylation of potential amino sugars was achieved by adding 200 μl of methanol, 20 μl of pyridine, and 20 μl of acetic anhydride to each unknown carbohydrate sample. The reaction was carried out for 30 min at room temperature. After the reaction period, the mixture was evaporated at 40 °C using a gentle stream of air. The carbohydrates were trimethylsilylated by adding 200 μl of the Tri-Sil HTP reagent (Thermo Scientific, Waltham MA.) to each sample. The mixture was incubated at 80 °C for 20 min before drying at room temperature under a gentle stream of air. The sample was dissolved in 1 ml of hexane and centrifuged at 1000 ×*g* for 5 min. The supernatant was transferred to a clean 2-ml autosampler vial and stored at −20 °C.

CPS analyses were carried out on a CP-3800 GC (Varian, Palo Alto, CA.) fitted with a Supelco SPB-608 30-m fused silica capillary column, containing a bonded stationary phase (proprietary phase, 0.25 μm film thickness). The TMS glycoconjugates were analyzed by mass spectrometry using the electron ionization mode with a Saturn 2200 GC/MS (Varian, Palo Alto, CA.). The initial oven temperature, of 80 °C, was held for 2 min. The temperature was raised to 160 °C by 20 °C/min and held at 160 °C for 12 min. The oven temperature was raised to 260 °C by 20 °C/min held at 260 °C for 7 min.

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

Additionally, *K. pneumoniae* capsules were visualized using Anthony's method (Hughes and Smith, 2007). Cultures of *K. pneumoniae* were grown for 24–48 h in LB broth containing the appropriate antibiotic. On a clean microscope slide, a 5 μL drop of the *K. pneumoniae* culture was mixed with a 5 μL drop fetal bovine serum (FBS). A 5 μL

drop of 1% crystal violet was added to the 10 µL mixture. The crystal violet was allowed to interact with the cells for 1 min before the mixture was smeared. The smear was then air dried for 10 min before the slide was gently washed with a 20% copper sulfate solution. The slide was dried on bibulous paper through capillary action. The cells were immediately observed using a brightfield microscope at 1000x.

2.4. Lipopolysaccharide quantification

Lipopolysaccharide (LPS) of whole cell bacteria was determined using the purpald assay (Turner et al., 2015; Velkov et al., 2013). This assay detects the 3-deoxy-D-manno-oct-2-ulopyranosonic acid (KDO) molecule of the LPS. Cultures were grown overnight, pelleted, and washed in 1X PBS; and 50 µL of the bacterial suspension was mixed with 50 µL of 32 mM sodium periodate. After 25 min of incubation at room temperature, 50 µL of 136 mM purpald in 2 N NaOH was added to each well of a 96 well plate and incubated for 20 min at room temperature. After incubation, 50 µL of 64 mM sodium periodate was added and incubated for 20 min at room temperature and absorbance was read at 540 nm A standard curve was generated using purified *K. pneumoniae* LPS (Sigma). 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 4 µg of LPS as determined by the purpald assay. Polysaccharide was stained using the Pro-Q Emerald 300 staining kit by Molecular Probes.

2.5. Transcription of outer membrane proteins

RNA was extracted from cultures in the exponential phase by treatment with RNA Protect, followed by extraction using the RNeasy kit with DNase treatment (Qiagen). Complimentary DNA (cDNA) was reverse transcribed from 1 µg 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 (Livak and Schmittgen, 2001). Gene expression was normalized to *gapA* and transcription levels in clinical isolates were normalized against CSUB10S + pSHA16 K. Primers used are listed in Table 1.

2.6. Bacterial interactions with macrophages

The murine macrophage cell line RAW 264.7 was maintained in 1640 RPMI media supplemented with 10% fetal bovine serum and penicillin, streptomycin, and amphotericin B at 37 °C in 5% CO_2. Macrophages were seeded into a 24 well tissue culture plate at 7.5×10^5 cells/mL and incubated overnight. Overnight bacterial cultures were suspended in a 1X PBS solution to contain approximately 1×10^9 CFUs/mL. Macrophages were treated with bacteria at a multiplicity of infection (MOI) of 50:1, a similar MOI to previous studies (Cano et al., 2015; March et al., 2013). Infection was synchronized using centrifugation at 200 ×*g* for 5 min, and samples were incubated at 37 °C for 15 min.

To determine bacterial attachment, macrophages were then washed with PBS to remove non-macrophage associated bacteria and lysed with 0.1% Triton X-100 in PBS. The lysate was serially diluted, plated on LB agar plates, and incubated overnight at 37 °C. The number of macrophage-associated bacteria was determined in CFU/mL. Percent association was determined by dividing the number of CFUs/mL recovered by the CFUs/mL of the original inoculum.

Macrophage production of reactive oxygen species (ROS) was indirectly determined by a modified nitroblue tetrazolium assay (Choi et al., 2006). Briefly, macrophages were seeded into the wells of a 96 well plate in 1640 RPMI antibiotic-free media as described above and bacteria were added and synchronized via centrifugation. Nitroblue tetrazolium (Fisher Bioreagents, Pittsburgh, PA.) was then added to a final well concentration of 0.1% and incubated for 60 min at 37 °C. After incubation, the supernatant was aspirated, and adherent cells were washed twice with warm PBS. Wells were then washed with methanol and air dried. The wells were solubilized with 2 M KOH and DMSO, and 100 µL of this sample was transferred to a new 96 well plate and read at 620 nm by a spectrophotometer. Untreated macrophage values were used as a negative control, and values were normalized against ROS production in response to strain 43816.

In order to determine the number of intracellular bacteria that survive phagocytosis, macrophages were infected at an MOI of 50:1 for 30 min, as described above. After incubation, the media was removed and replaced with media containing either 500 µg/mL gentamicin (clinical isolates) or 200 µg/mL gentamicin (43816) for 2 h at 37C. The number of bacteria surviving phagocytosis was then determined by lysing macrophages with 0.1% Triton X-100; serially diluting the lysate, and plating on LB agar plates.

We further enumerated the total rate of survival by phagocytosed bacteria, to include those that escape from within macrophages. Briefly, macrophages were seeded and infected as described above and incubated for 30 min. After 30 min, the media was removed and replaced with media with gentamicin for an additional 30 min. This eliminated all extracellular bacteria. The macrophages were then washed three times with 1X PBS and incubated in antibiotic free media for two hours at 37C. The total number of bacteria in the well was then determined by lysing remaining macrophages and supernatant with 0.1% Triton X-100; serially diluting the lysate, and plating on LB agar plates. Bacterial survival was then calculated as the ratio of CFU between the two hour incubated cells and the cells immediately after gentamicin treatment.

2.7. Statistical analysis

All experiments were performed with $n \geq 3$. Statistical significance was determined using a one-way ANOVA and Tukey's post hoc test using XLSTAT software. Significance for all experiments were compared against clinical strain CSUB10S + 16 K, which expressed both porins. For the ROS analysis, responses were compared to that of lab strain

Table 1
Primers used for qPCR.

	Primer Sequence (5′ –→ 3′)	
Bacterial 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

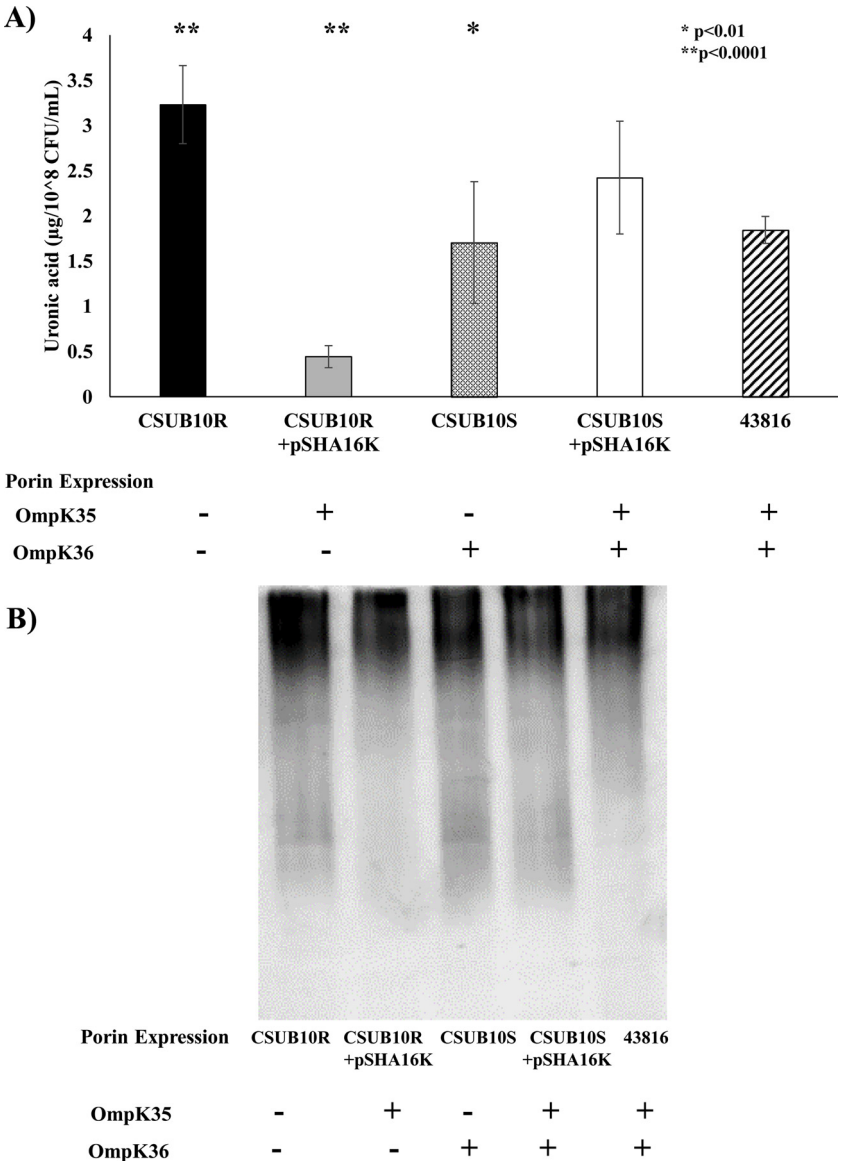

Fig. 1. Porin loss alters production of capsule. A) Capsule production was quantified by glucuronic acid assay and normalized per 10^8 CFU ($n \geq 9$). Bars represent one standard deviation from the mean. Statistical comparisons are all made to strain CSUB10S + 16 K. Significance was determined at p-values of < 0.01 (*) and $p < 0.0001$ (**). B) Visualization of capsule on 12% SDS-PAGE stained with Pro-Q Emerald Polysaccharide stain.

43816. Significance was determined at p-values of < 0.01 or less.

3. Results

3.1. Capsule production is altered in porin loss strains

In order to investigate the impact of porin loss on bacterial physiology and production of virulence factors, we utilized a collection of clonally related, ESBL positive strains of *Klebsiella pneumoniae*. These strains exhibit sequential loss of OmpK35 and OmpK36. The strains were further transformed with plasmid expressed porins containing a native promoter to reconstitute a strain expressing both porins and a strain expressing only OmpK35. These strains have been previously characterized and determined to have no significant differences in their bacterial growth and outer membrane integrity (Turner et al., 2016). Therefore, this panel of different combinations of porin expression are representative of host of changes that may occur through natural selection in a clinical setting.

We first evaluated the impact of porin loss on production of capsule, the best characterized virulence factor of *K. pneumoniae*. Capsular polysaccharide was extracted, and the production of capsule was quantified by glucuronic acid concentration. Glucuronic acid is a major component of *K. pneumoniae* capsules, and therefore can be used as an indirect measurement of overall capsule content. Statistical comparisons were made against strain CSUB10S + pSHA16k, rather than strain 43816 because the ATCC lab strain is a different capsular serotype than that of the clinical isolates. Therefore, the reference strain was chosen to minimize the possible effect of different capsular serotypes on results.

Both CSUB10R + pSHA16 K and CSUB10S, which exhibit loss of only one porin (OmpK36 and OmpK35, respectively), produced significantly less capsule than CSUB10S + pSHA16 K, which expresses both porins (Fig. 1A). The capsular polysaccharides were further visualized by SDS-PAGE to determine differences in molecular weight distribution (Fig. 1B). The goal of this analysis was to determine whether the changes observed in capsule production were due to changes in polysaccharide chain length, or overall changes in capsule production. As seen in Fig. 1B, separation of the capsule on SDS-PAGE revealed that

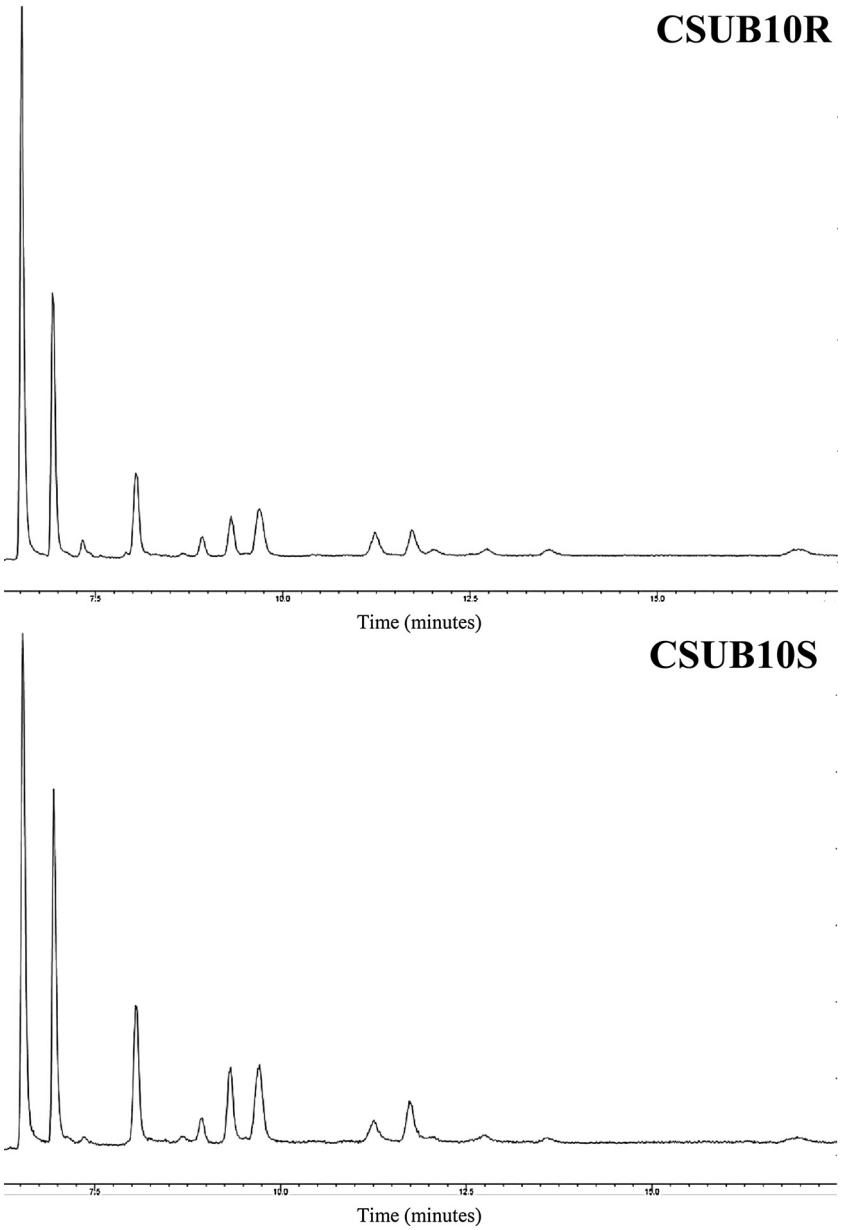

Fig. 2. Porin Loss does not significantly alter capsule carbohydrate composition. GC–MS analysis of the carbohydrate composition of the capsules of CSUB10S and CSUB10R.

strain CSUB10R + 16 K exhibited only a slight decrease in staining for low molecular weight polysaccharides. This suggested that the effect was likely due to a decrease in overall capsule production and not in polysaccharide chain length.

To further determine the effect of porin loss on capsule composition, the hydrolyzed CPS extracts were analyzed by GC–MS. As seen in Fig. 2, both strains CSUB10S and CSUB10R had identical chromatograms, which indicated that the carbohydrates in each capsule were identical. PCR analysisdemonstrated that the strains were not of the most common K1, K2, or K5 capsule serotypes (data not shown). Regardless, the GC–MS analysis demonstrated that the two clonally related strains were the same serotype, and that the significant changes in capsule production were not due to changes to the carbohydrate composition of the capsule.

Observations of the colony morphology and capsule stains further demonstrated changes in capsule production. Colonies from strain CSUB10R + pSHA16 K were visibly smaller and less mucoid (Fig. 3A). Staining and visualization of the capsule (Fig. 3B) also revealed a less distinct capsule halo surrounding cells in strain CSUB10R + 16 K compared to other isolates.

While loss of either one porin decreased capsule content, strain CSUB10R, which lacked both OmpK35 and OmpK36, exhibited a statistically significant increase in capsule production as quantified by the uronic acid assay (Fig. 1A). This quantitative increase in capsule was not significantly reflected in either the SDS-PAGE or the stained images. Given the GC–MS analysis, this change cannot be explained by a change in capsule composition. Together, these data indicate that capsule quantity may be actively regulated in response to changes in porin expression.

3.2. LPS content is increased with porin loss

Previous work with these bacterial strains demonstrated that porin loss did not alter overall membrane integrity (Turner et al., 2016). In order to investigate changes to the overall quantity of LPS in the cell, cellular LPS content was determined by the purpald assay, which

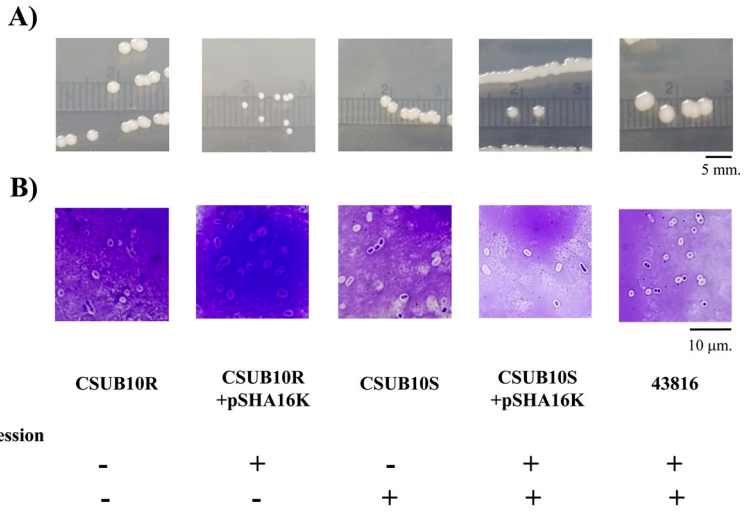

Fig. 3. Visualization of Capsule in Porin Loss strains A) Colony morphology of strain grown for 24 h on LB media with appropriate antibiotics. Scale bar is 5 mm. B) Staining of encapsulated cells. Cells were stained using Anthony's method, and visualized at 1000X total magnification. Scale bar is 10 µm.

directly detects the KDO molecule within the LPS polymer. The average cellular LPS content was significantly increased in strains with loss of one or both porins (CSUB10S, CSUB10R + pSHA16 K, and CSUB10R) relative to the isolate that expressed both porins (CSUB10S + pSHA16 K) (Fig. 4A).

While the purpald assay can be used to determine overall LPS quantity, it did not account for possible changes in the polysaccharide length of the LPS molecule. Therefore, the LPS from purified outer membranes was also visualized by SDS-PAGE to detect alterations in polysaccharide lengths (Fig. 4B). This revealed that the LPS of the clinical isolates had a lower molecular weight than LPS isolated from ATCC lab strain 43816. The presence of rougher LPS may impact the inflammatory response of exposed macrophages when compared to the reference strain (ATCC 43816). Additionally, the isolate CSUB10R + pSHA16 K also showed a visible decrease in LPS staining, which correlated with low capsule content of this strain. One explanation of the light staining is an alteration to the O-antigen of LPS. Together, the LPS and capsule data suggested that loss of only OmpK36 may impact both LPS and capsule composition and production.

3.3. Loss of porins OmpK35 and OmpK36 trigger increased expression of compensatory porins

K. pneumoniae strains with loss of OmpK35 and OmpK36 have been observed to express other alternative porins (Garcia-Sureda et al., 2011a, b). Therefore, the expression of alternative porins with more restrictive pore sizes was analyzed. SDS-PAGE and qPCR were used to determine changes in porin expression that correlated with loss of OmpK35 and/or OmpK36. Relative transcriptional levels were determined using the ΔΔCt method comparing against the housekeeping gene *gapA* and target gene expression in the strain CSUB10S + 16k. Statistical comparisons were made to the same clinical strain expressing both OmpK35 and OmpK36. The transcriptional levels of each porin relative to the clinical isolates indicate the fundamental differences between lab and clinical strains.

Transcription of genes that aide in the structural integrity of the cell envelope, such as *ompA* and *lpp*, were quantified to investigate whether porin loss required compensation to maintain overall integrity of the cell envelope. The transcriptional expression of *ompA* (Fig. 5A) was decreased with the loss either one porin, but not affected by the loss of both porins, in comparison to the isolate that expressed both porins. This was reflected in the SDS-PAGE as well (Fig. 6). In contrast, transcription of *lpp* (Fig. 5B) showed significant changes in expression depending on the porin expression profile. Loss of both porins (CSUB10R) resulted in a decrease in *lpp* transcription, while the loss of OmpK36 (CSUB10R + pSHA16 K) triggered a highly significant increase in transcription. These data indicate that porin loss triggered gene specific changes in outer membrane protein expression to maintain outer membrane stability.

The outer membrane porins OmpK26 and LamB have been previously characterized as less permeable to β-lactam antibiotics than OmpK35 or OmpK36 (Garcia-Sureda et al., 2011a, b). Therefore, both porins were ideal candidates as possible alternatives for the compensation of nutrient acquisition when OmpK35 and/or OmpK36 were lost. Transcriptional analysis of *ompK26* (Fig. 5C) showed significant increases in expression in all porin loss strains, but most significantly in those strains lacking OmpK35. Transcription of *lamB* (Fig. 5D) was significantly increased with the loss of only OmpK36 (CSUB10R + pSHA16 K). It is likely that loss of OmpK35 or both porins and increased expression of OmpK26 is more beneficial than the loss of OmpK36 alone with increased expression of LamB. Clinical isolates exhibiting loss of OmpK36 alone are extremely rare (Dara et al., 2014; Hong et al., 2013; Lee et al., 2016; Zhang et al., 2014). The results of these transcriptional analyses are summarized in Table 2. Outer membrane proteins were also visualized by SDS-PAGE (Fig. 6). This gel shows porin loss in each strain and expression of alternative porins. Together, these data illustrate that loss of OmpK35 and OmpK36 resulted in a pattern of up-regulation of specific unique compensatory porins in the presence of β-lactam antibiotics.

3.4. Loss of OmpK36 alone significantly decreases pathogenicity of Klebsiella pneumoniae

Phagocytosis assays were performed to determine how porin loss alters bacterial interactions with macrophages and levels of macrophage activation. The number of macrophage associated bacteria was determined as an indirect indication of the rate of phagocytic uptake. The percent attachment reflects the proportion of the initial inoculum that are surface associated with or internalized by macrophages after a standard 15-minute incubation. As seen in Fig. 7A, loss of either porin resulted in a significant increase in association with phagocytic cells which notably also correlated with a decrease in capsule. However, the loss of both OmpK35 and OmpK36 (CSUB10R) resulted in a similar level of attachment when compared to the lab strain and the clinical strain which expressed both porins. This could be due to the increased capsule surrounding CSUB10R, as the capsule likely provides some protection against attachment by the macrophages.

Effective killing of phagocytosed bacteria requires macrophage

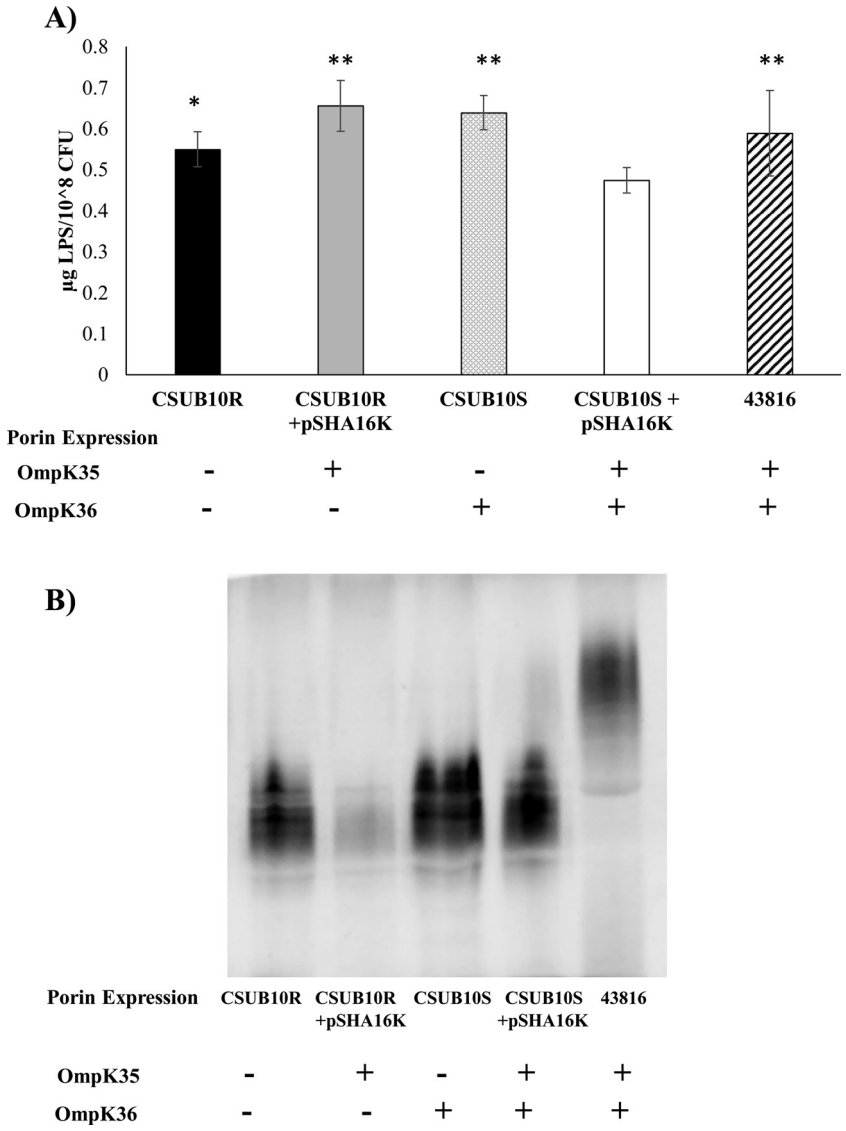

Fig. 4. LPS content is increased with porin loss. A) Whole cell LPS was quantified by the Purpald Assay. LPS content was normalized to μg LPS/ 10^8 CFU. n = 18 from 3 independent cultures. Bars represent one standard deviation from the mean. n ≥ 3 for each culture. Statistical comparisons are to strain CSUB10S + 16 K. Significance was determined at p-values of < 0.01 (*) and p < 0.0001 (**). B) Visualization of isolated LPS on 12% SDS-PAGE gel stained with Pro-Q Emerald Polysaccharide stain.

activation and production of bactericidal products. The activated, bactericidal response of macrophages in response to *Klebsiella* was determined by measuring reactive oxygen species (ROS) production by a modified nitroblue tetrazolium assay (Choi et al., 2006). Fig. 7B shows that all bacterial strains tested triggered nitric oxide production. ROS production was normalized against the most intense response to strain 43816. All isolates exhibiting loss of one or more porin triggered significantly less ROS production than that to strain 43816. The clinical strain expressing both porins (CSUB10S + pSHA16k) triggered more ROS production than the other clinical strains, but still less than to lab strain 43816. This may indicate that porins themselves are significant triggers of ROS production, dampened in part by increased capsule production in the clinical isolates.

Recent studies have indicated that *Klebsiella* may be able to survive phagocytosis and even escape from the phagolysosome (Cano et al., 2015). Therefore, one goal of this study was to assess the ability of internalized *Klebsiella* to survive bacterial phagocytosis. First the number of intracellular bacteria that survived a period of phagocytosis was determined by killing all remaining extracellular bacteria via gentamicin treatment. As seen in Fig. 7C, each clinical isolate exhibited the ability to survive within phagocytic cells. While not statistically significant, strain CSUB10R, which exhibits dual porin loss, had the highest rate of intracellular survival.

The ability of *Klebsiella* to not only survive in an intracellular environment, but to escape the phagocytic cells and proliferate in the culture media was then investigated. For this assay, macrophages were initially exposed to bacteria as previously described for 30 min. Extracellular bacteria were then removed by treatment with gentamicin and washing. This initial inoculum of internalized bacteria was determined by plate count. Macrophages with internalized bacteria were then incubated for two hours without gentamicin. This allowed for survival of any bacteria that could exit macrophages back into the culture supernatant, and the change in the number of bacteria from the starting internalized inoculum was determined as a ratio. As seen in Fig. 7D, this value represents the ability of each of these strains to survive and escape from a phagocytic cell.

Each clinical isolate was able to survive and proliferate after phagocytosis, while the lab strain 43816 did not. Significantly, the two strains best able to survive and proliferate were the two naturally occurring strains CSUB10R and CSUB10S. The large increase in bacteria

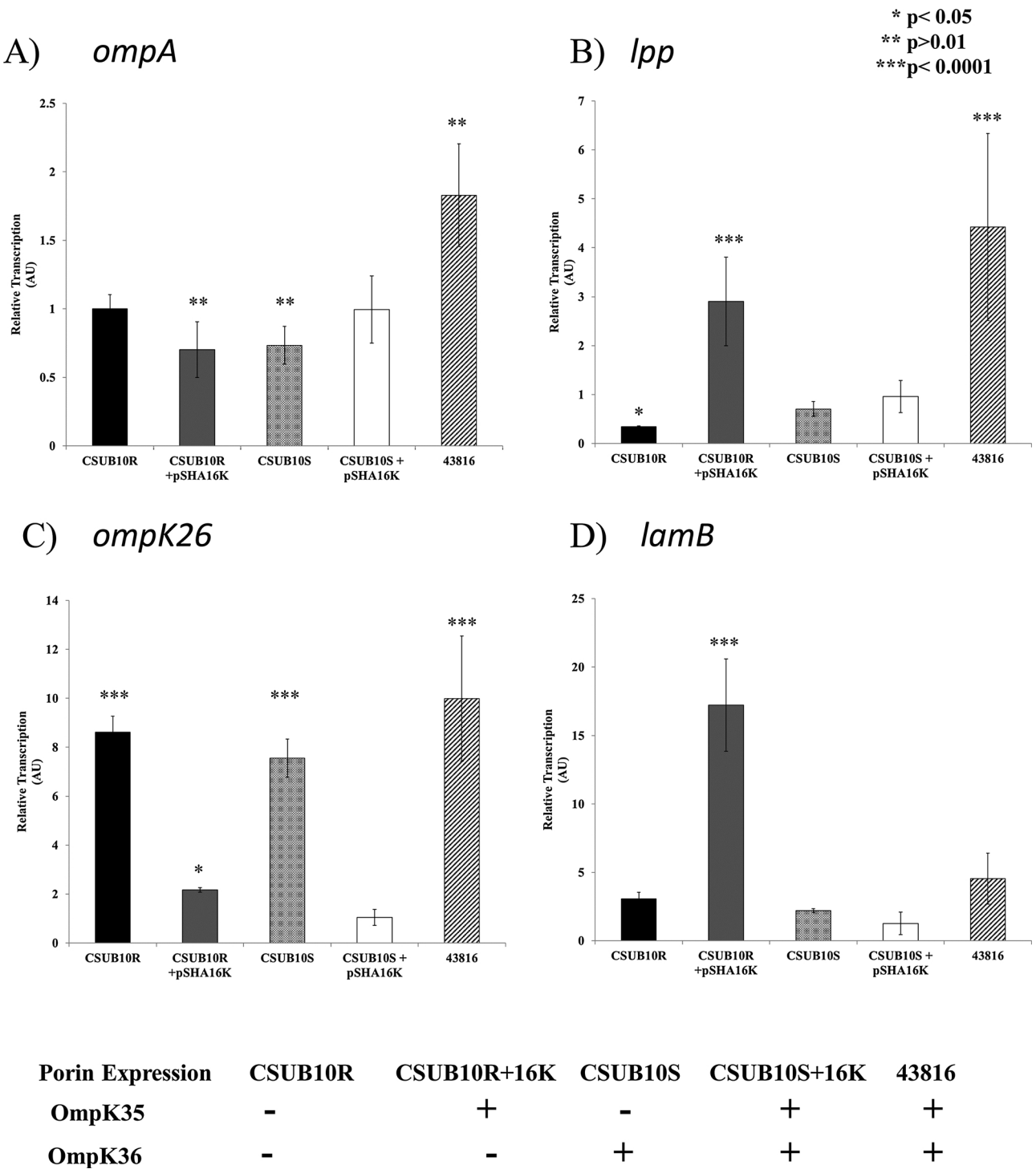

Fig. 5. Transcription of alternative porins. Transcription of the alternative porins A) *ompA*, B) *lpp*, C) *ompK26*, and D) *lamB* was determined by qPCR. Relative transcriptional levels determined using the ΔΔCt method comparing against the housekeeping gene *gapA* and target gene expression in strain CSUB10S + pHSA16 K. n ≥ 7 from at least two independent cultures. Bars represent one standard deviation from the mean. Statistical comparisons are all compared against strain CSUB10S + 16 K. Significance was determined at p-values of < 0.05 (*), p < 0.01 (**), and p < 0.0001 (**).

over the two-hour period indicated that the CSUB10R and CSUB10S strains were more efficient at survival or escape from the macrophages. This data shows that the survival and escape of *K. pneumoniae* from macrophage killing is impacted by but not completely dependent on porin loss, because each clinical isolate tested demonstrated some degree of escape and extracellular replication.

4. Discussion

The goal of this study was to characterize the impact of antibiotic resistance-associated porin loss on the major virulence factors of *K. pneumoniae*. We found that expression of only one non-specific porin, either OmpK35 or OmpK36, was paired with decreased capsule production, increased membrane LPS content, and higher levels of binding and internalization by macrophages. In contrast, halted expression of

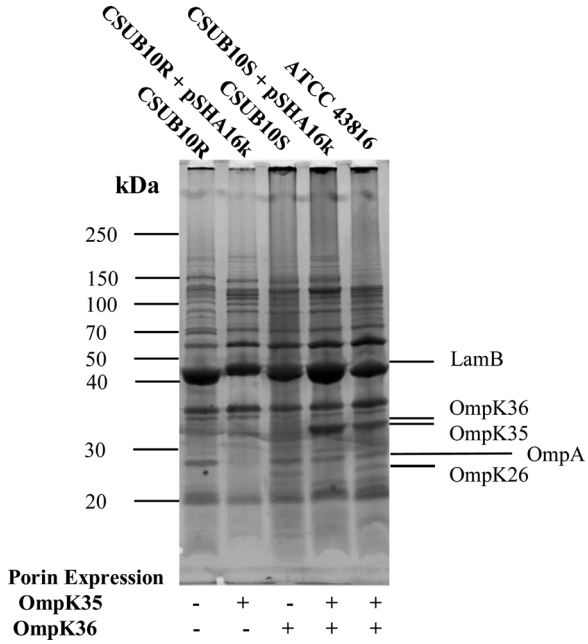

Fig. 6. Porin loss strains exhibit expression of alternative porins. 12% SDS-PAGE of purified outer membrane proteins from each strain. Arrows indicate the positions of LamB, OmpK35, OmpK36, and OmpK26, and OmpA.

Table 2
Changes in Porin Transcription as a Result of Antibiotic Associated Porin Loss.

	CSUB10R	CSUB10R +pSHA16K	CSUB10S	CSUB10S +pSHA16K
ompK35	–	+	–	+
ompK36	–	–	+	+
ompA	NC	–	–	NC
lpp	–	+ +	NC	NC
ompK26	+ +	+	+ +	NC
lamB	NC	+ +	NC	NC

both OmpK35 and OmpK36 resulted in highly elevated capsule production that likely contributed to the evasion of phagocytosis and to the ability of this strain to dampen the bactericidal response of macrophages, resulting in greater bacterial survival. Loss of porins was also clearly paired with the up-regulated expression of specific alternative porin genes. Together, these data strongly indicate that the evolution of a porin loss strain is associated with cumulative changes in production of multiple virulence-associated factors, which promote the survival of the bacteria in the antibiotic treated host.

The bacterial capsule is the best studied virulence factor of *Klebsiella*. This secreted layer of polysaccharides has been clearly linked with bacterial evasion of the immune system, and acapsular mutants have been found to have highly reduced virulence (Domenico et al., 1985; Kabha et al., 1995; Paczosa and Mecsas, 2016). Therefore, our initial hypothesis was that the capsule would be maintained or enhanced in porin loss isolates. This result was confirmed in the dual porin loss strain. However, expression of only one of either porin genes resulted in a decrease in capsule production. This decrease concurs with the work of Srinivasan *et al.* (Srinivasan et al., 2012), who found that isogenic deletion of an OmpK36 homologue both altered colony morphology and significantly reduced production of capsular glucuronic acid. A different study utilizing isogenic mutants also found that loss of either OmpK36 alone or both OmpK35 and OmpK36 resulted in a more mucoid colony morphology (Tsai et al., 2011). The analysis of capsule composition by GC–MS presented here indicates that these changes are likely not due to alterations in carbohydrate composition, but rather changes in the total amount of capsule.

Klebsiella has at least 77 different capsular serotypes (Kabha et al., 1995). It is therefore possible that the impact porin loss has on the production of capsule could be dependent on serotype. The capsule serotypes of the clinical isolates used in this study (CSUB) were determined by PCR to not be of the most common K1, K2, or K5 (Turton et al., 2008) (data not shown), and capsule composition did not change with changes in porin expression. However, our data indicates that the effect of porin loss in these clinical isolates is similar to the mutants created in a K1 serotype strain seen by Srinivasan et al. (Srinivasan et al., 2012). It is unknown whether porins are responsible for the uptake of the specific monomeric sugars required 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. However, the significantly increased capsule production observed in the dual porin loss strain does not support this hypothesis.

Lipopolysaccharide is an essential component of the Gram-negative outer membrane, a major activator of the innate inflammatory system, and a primary ligand of the complement cascade. The primary site of variation within the LPS molecule is the outer O-antigen, which functions to shield the bacterial outer surface from binding and recognition by immune components. Our analysis of LPS content and composition revealed that porin loss in general triggered increased LPS content in the bacterial outer membrane. Our previous work has demonstrated that these porin loss strains do not exhibit a loss in membrane integrity, and this increase in LPS content may be a compensatory mechanism to achieve this stability (Turner et al., 2016). A more striking difference was observed in the size of the LPS molecules as visualized by SDS-PAGE. All of the clonally related clinical isolates produce much smaller LPS molecules, indicative of a rough variant of LPS.

Rough LPS exhibits truncated or absent O-antigen sugars. Loss of the O-antigen has been shown to be irreversible in some strains and results in increased sensitivity to complement killing and phagocytosis (Hansen et al., 1999; Hsieh et al., 2012; Shankar-Sinha et al., 2004). Our clinical isolates did exhibit a greater rate of attachment to macrophages than that of the lab strain 43816, which has a smooth type LPS. However, the clinical isolates were able to survive or evade the killing mechanisms of phagocytosis. These data indicate that LPS content may be more critical for the maintenance of membrane integrity than immune evasion.

The analysis of porin transcription patterns concurs with previous observations that porin loss is paired with up-regulation of alternative beta-barrel pore proteins. Both *ompK26* and *lamB* have been observed to have increased expression in porin loss strains as seen by bands in SDS-PAGE gels (Garcia-Sureda et al., 2011a, 2011b). The current study is distinctive because it analyzed specific gene transcripts across the panel of clonally related strains with different expression patterns of OmpK35 and OmpK36. Together, these data confirm that loss of OmpK35 is paired with increased *ompK26*, while loss of OmpK36 is paired with increased *lamB*. Notably, loss of both porins resulted in very high levels of *ompK26* transcripts, but not significant change in *lamB*. Analysis of specific gene transcription is a more accurate measurement than evaluating the density of bands on an SDS-PAGE gel. However, post-transcriptional control of protein synthesis can modulate large changes seen at the transcriptional level resulting in more modest changes in band intensity on a gel.

This observation is in agreement with previous research, which found that the expression of OmpK26 was essential for compensation of the loss of OmpK36. Deletion of the *OmpK26* gene was only possible in strains expressing OmpK36 (Garcia-Sureda et al., 2011a). Likewise, deletion of *lamB* in a dual porin loss background had been shown to result in increased OmpK26 expression (Garcia-Sureda et al., 2011b). This demonstrates that OmpK26 may be indispensable in a dual porin loss strain, and that there are limited gene options for replacing the essential physiologic functions of these nonspecific transmembrane pores.

Porin loss also resulted in changes in the expression of *ompA* and *lpp*,

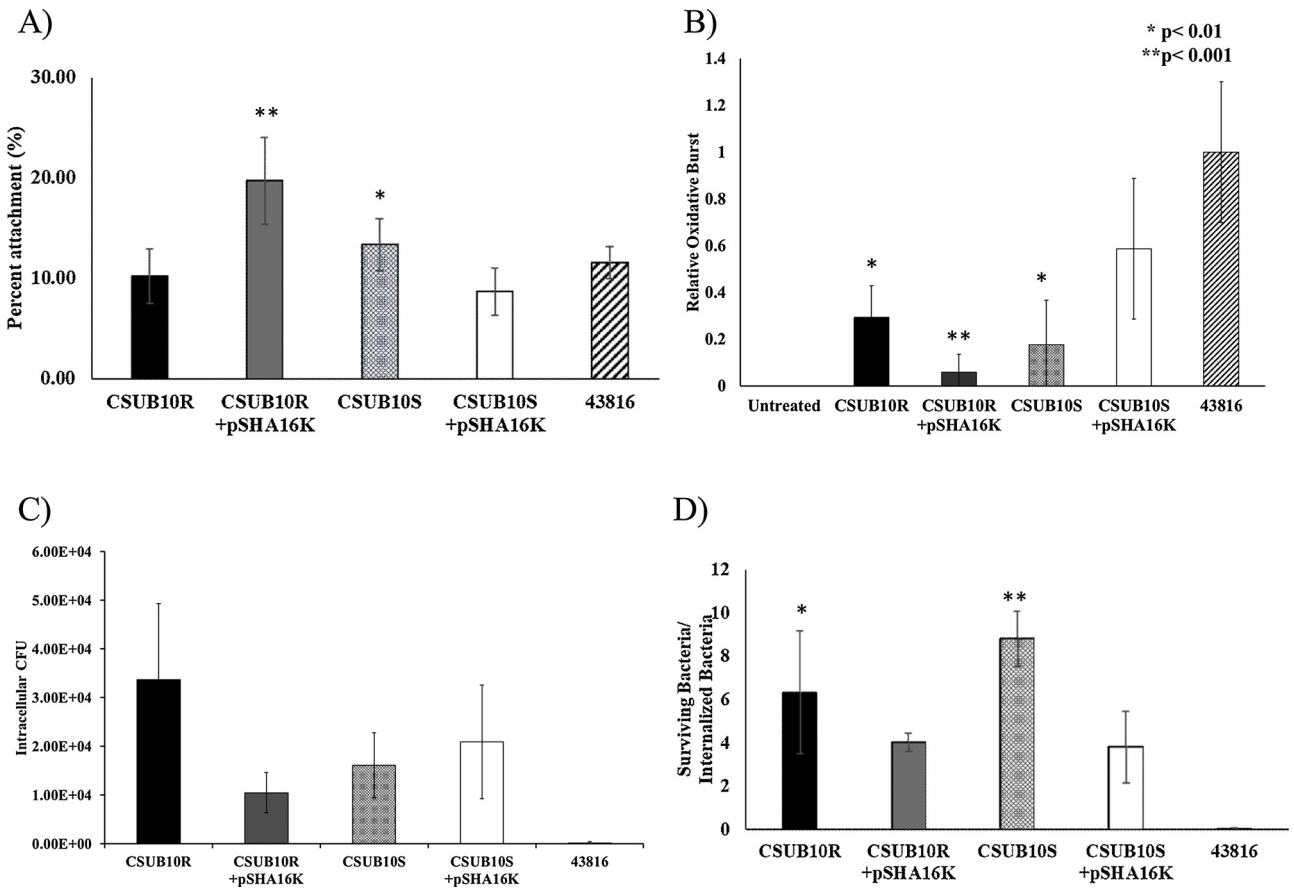

Fig. 7. Porin expression impacts bacterial survival of phagocytosis. A) Attachment and uptake of bacteria by macrophages. Bacteria were incubated with macrophages for 15 min at an MOI of 50:1, and total adherent or internalized bacteria were enumerated. Statistical comparisons are to strain CSUB10S + 16 K. B) Reactive Oxygen species (ROS) production of macrophages in response to porin loss strain. ROS production by macrophages was determined by a Nitroblue Tetrazolium assay after a one-hour incubation with bacteria at an MOI of 50:1. Values were normalized against the reaction to strain 43816. C) Intracellular survival of bacteria after phagocytosis. Bacteria were incubated with macrophages for 30 min at an MOI of 50 :1, washed and treated with media with gentamicin for 2 h. Surviving intracellular bacteria were determined by plate count. D) Total survival of bacteria after phagocytosis. Bacteria were incubated with macrophages for 30 min at an MOI of 50 :1, washed and briefly treated with gentamicin to eliminate extracellular bacteria. Cultures were then incubated without gentamicin for two hours. The number of total surviving bacteria from this internalized inoculum was then determined by plate count. The ratio of the total bacteria recovered to the initial internalized CFU was determined. Statistical comparisons are to strain CSUB10S + 16 K. For all experiments in this figure: $n \geq 9$, bars represent one standard deviation from the mean. Significance was determined at p-values of < 0.01 (*) and $p < 0.001$ (**).

which are critical to the maintenance of outer membrane stability. There is extensive work demonstrating that deletion of OmpA has a negative effect on bacterial virulence and survival within the host (March et al., 2013, 2011). Studies of the role of *lpp* in pathogenesis are more limited but indicate that the deletion of *lpp* contributes to greater sensitivity to killing by both phagocytosis and complement (Hsieh et al., 2013). However, deletion of either of these genes also results in disruption of outer membrane stability, rendering the bacteria sensitive to bile salts and detergents. Therefore, the association of these proteins with virulence is more likely a requirement to maintain a robust cellular envelope than a direct interaction of these proteins with host components.

The data presented here supports the emerging evidence that *K. pneumoniae* has the capacity to survive and escape phagocytosis (Cano et al., 2015). Our data suggests that clonally related isolates all exhibit a significant ability to survive macrophage exposure compared to the lab strain 43816. Of our clonally related strains, the greatest rate of survival can be paired with high levels of capsule production and a greatly reduced oxidative burst. These data concur with previous studies that document *Klebsiella* establishing a vacuole within macrophages that does not receive lysosomal enzymes (Cano et al., 2015). A study of the inflammatory response of epithelial cells further indicates that *Klebsiella* has the capacity to actively suppress host inflammatory signaling (Regueiro et al., 2011). Of note, both of these studies demonstrate that capsule is not the cellular component driving either the suppression of inflammatory cytokine production or the evasion of phagolysosome formation. Our data may then suggest that components such as LPS O-antigen and porin expression may contribute to these mechanisms of bacterial survival in the host.

It is notable that the strains that were best able to survive phagocytosis were also the two strains directly isolated from the clinical setting. This finding corresponds with the rarity of seeing porin loss isolates that have only lost OmpK36 (Shakib et al., 2012). It is also likely that during infection of an antibiotic treated host, loss of OmpK35 occurs first with the subsequent loss of OmpK36 occurring only after prolonged treatment with antibiotics (Domenech-Sanchez et al., 2003; Hernandez-Alles et al., 1999). In the current study, creation of this rare single porin expression profile was further paired with low capsule expression and high expression of *lpp*. While this strain did survive macrophage phagocytosis in vitro, these experiments do not account for changes in sensitivity to complement binding or opsonization, which have been previously shown to be affected by changes in porin and capsule expression (Alberti et al., 1993, 1995; Domenico et al., 1985; Merino et al., 1992). Studies of isogenic deletion mutants of these porins indicate that loss of either OmpK35 or OmpK36 should decrease bacterial survival and fitness both in vitro and in vivo (Chen et al.,

2010; Tsai et al., 2011). However, these isogenic studies did not investigate the downstream impact on expression of other virulence factors. Further investigation is needed to determine if the absence of outer membrane porins are directly responsible for changes in bacterial virulence.

In summary, this study demonstrated that antibiotic resistance associated with porin loss was accompanied by a suite of changes in capsule, LPS, and expression of alternative porins. These changes act to nullify any negative fitness effect due to lack of the nonspecific porin proteins, allowing the bacteria to grow and survive phagocytic immune responses. This analysis further demonstrates that expression of virulence factors cannot be analyzed in isolation, but rather each individual component may contribute to the overall virulence of the organism as a whole. *Klebsiella* has frustrated many due to the lack of clear virulence determinants, such as toxins or secretion systems (Broberg et al., 2014; Paczosa and Mecsas, 2016). This study adds to the increasing evidence that the main pathogenic mechanism of *K. pneumoniae* is one of evasion, mediated by a combination of capsule, LPS, outer membrane porins and other less traditional virulence-associated factors.

Competing interests

The authors declare that they have no competing interests.

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