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Porin Loss Impacts the Host Inflammatory Response to Outer Membrane Vesicles of *Klebsiella pneumoniae*

Kelli L. Turner,[a] Bethaney K. Cahill,[a] Sarah K. Dilello,[a] Dedra Gutel,[a] Debra N. Brunson,[a] Sebastián Albertí,[b] Terri N. Ellis[a]

Department of Biology, University of North Florida, Jacksonville, Florida, USA[a]; IUNICS, University of the Balearic Islands, Palma de Mallorca, Spain[b]

Antibiotic-resistant strains of *Klebsiella pneumoniae* often exhibit porin loss. In this study, we investigated how porin loss impacted the composition of secreted outer membrane vesicles as well as their ability to trigger proinflammatory cytokine secretion by macrophages. We hypothesize that porin loss associated with antibiotic resistance will directly impact both the composition of outer membrane vesicles and their interactions with phagocytic cells. Using clonally related clinical isolates of extended-spectrum beta-lactamase (ESBL)-positive *Klebsiella pneumoniae* with different patterns of porin expression, we demonstrated that altered expression of OmpK35 and OmpK36 results in broad alterations to the protein profile of secreted vesicles. Additionally, the level of OmpA incorporation was elevated in strains lacking a single porin. Porin loss significantly impacted macrophage inflammatory responses to purified vesicles. Outer membrane vesicles lacking both OmpK35 and OmpK36 elicited significantly lower levels of proinflammatory cytokine secretion than vesicles from strains expressing one or both porins. These data demonstrate that antibiotic resistance-associated porin loss has a broad and significant effect on both the composition of outer membrane vesicles and their interactions with phagocytic cells, which may impact bacterial survival and inflammatory reactions in the host.

Klebsiella pneumoniae is a nosocomial pathogen responsible for as many as 20% of all cases of culture-positive blood cultures and bacterial sepsis (1). Alarmingly, up to 50% of these infections are resistant to most current antibiotics (2, 3). The majority of resistant *Klebsiella* isolates exhibit both expression of an extended-spectrum beta-lactamase (ESBL) and halted expression of at least one outer membrane porin (4, 5). Porin loss has been clearly shown to enhance levels of antibiotic resistance, and ESBL-positive *K. pneumoniae* isolates commonly exhibit loss of OmpK35 and OmpK36, which both function in nonspecific transport across the outer membrane (6–8).

While changes to the porin profile of bacteria are well documented to be associated with enhanced antibiotic resistance (9, 10), the role that these porins play in pathogenesis has not been fully investigated. *In vitro* experiments using mutants of *K. pneumoniae* lacking OmpK36 have demonstrated that loss of OmpK36 results in increased phagocytic killing of the bacteria, and *in vivo* experiments have demonstrated decreased virulence and bacterial survival of strains lacking OmpK36 in a mouse model (11–14). Data from these studies consistently indicate that loss of OmpK36 results in an increase in antibiotic resistance paired with a decrease in fitness and ability to survive host immune responses. It is unclear how resistant strains of *Klebsiella* are able to persist in host tissues when porin loss renders the bacteria more susceptible to phagocytic clearance.

The capsule is the best understood of the virulence factors of *Klebsiella*, cloaking the bacterial outer surface from immune recognition and inhibiting phagocytic cell uptake. However, the roles of other secreted factors in the virulence of this organism have not been well defined. These secreted factors, such as outer membrane vesicles (OMVs), may play a key role in regulating the inflammatory response to this infection. OMVs are naturally secreted from all Gram-negative bacteria. The protein and lipid contents of secreted vesicles are derived from the outer membrane and can be enriched with proteins from the membrane or the periplasmic space. These enriched proteins include porins and other proteins known to impact host cell functions (15–17). OMVs are particularly effective at delivering diverse cargo to host cells, effecting responses that range from inflammatory activation to intoxication and cell death. Vesicles have also been shown to inhibit macrophage phagosome-lysosome fusion, modify host cell secretion, and stimulate intense inflammatory responses. The production of vesicles has been shown across a broad range of Gram-negative pathogens to be an essential physiological function of bacteria, including survival within infected human tissues (17, 18).

It has been demonstrated by our lab and others that *K. pneumoniae* strains produce vesicles that are laden with gene products associated with virulence and are capable of triggering a potent inflammatory response (19, 20). Lee et al. were the first to broadly characterize *Klebsiella* OMV protein composition. They identified over 150 protein components, including OmpA, SlyB, and NlpD, which are all associated with bacterial adhesion and virulence. They further demonstrated that purified vesicles trigger inflammatory responses both *in vitro* and *in vivo* (19).

In this study, we hypothesize that porin loss associated with antibiotic resistance will directly impact both the composition of outer membrane vesicles and their interactions with phagocytic cells. Porins are known major components of outer membrane vesicles, and OMVs have been shown in a number of bacterial species to be a potent stimulator of innate inflammatory signaling (17, 19). Using clonally related clinical isolates of ESBL-positive

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Address correspondence to Terri N. Ellis, terri.ellis@unf.edu.

Klebsiella pneumoniae, we demonstrate that porin loss does not impact cellular growth, membrane integrity, or OMV yield. However, altered expression of OmpK35 and OmpK36 does result in larger-scale alterations to the protein profile of secreted vesicles of these clinical isolates. Furthermore, porin loss had a significant impact on macrophage inflammatory responses to OMVs from these strains. Outer membrane vesicles lacking both OmpK35 and OmpK36 elicited significantly lower levels of proinflammatory cytokine secretion than did vesicles from strains expressing one or both porins. Based on our findings, we conclude that antibiotic resistance-associated porin loss has a broad and significant effect on both the composition of outer membrane vesicles and their interactions with phagocytic cells, which may impact bacterial survival and inflammatory reactions in the host.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Klebsiella pneumoniae* isolates 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 (8, 21). Strain 10S expresses only OmpK36, while strain 10R expresses neither OmpK35 nor OmpK36. Strain CSUB10R was further transformed with either plasmid pSHA16k, which contains *ompK35*, or pSHA21, which contains *ompK36* (8). Strain CSUB10S was also transformed with plasmid pSHA16k to create a strain that expresses both OmpK35 and OmpK36 porins. All cultures were grown in LB broth with 16 μg/ml cephalothin, and strains with plasmids were grown in cephalothin and 50 μg/ml kanamycin. Cultures were grown at 37°C with agitation (200 rpm), and growth was determined by spectrophotometry at 600 nm.

Western blot analysis. Integrity of the bacterial cell was determined by Western blot assay of the whole-cell lysate (WCL) and concentrated culture supernatant for the cytoplasmic protein RecA. Cell pellets were lysed by sonication in phosphate-buffered saline (PBS) at 4°C. Culture supernatants were concentrated using Amicon Ultracell 10-kDa-cutoff centrifugal filter units (EMD Millipore) to a final protein concentration of >200 μg/ml as determined by the Bradford assay (Coomassie Plus; Thermo Pierce). Ten micrograms of total protein of whole-cell lysate and culture supernatant was separated by 12% SDS-PAGE and transferred to nitrocellulose. Membranes were blocked with 5% nonfat dried milk in PBS-Tween 20 (PBST), probed with primary antibodies against rabbit anti-RecA (PA-4925; Thermo), and detected using goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (Sigma). Blots were developed using the Thermo 1-step TMB (3,3′,5,5′-tetramethylbenzidine) reagent.

Membrane permeability assay. Integrity of the bacterial membrane was further determined by a disc diffusion assay described by Llobet et al. (22). Sterile filter discs containing either 100 μg of SDS or 80 μg of novobiocin were overlaid on lawns of each bacterial species. Plates were incubated overnight at 37°C, and the diameter of the zone of inhibition was measured in millimeters.

Outer membrane vesicle purification. Cultures (250 ml) of *K. pneumoniae* strains were grown for 12 h, and the bacterial cells were pelleted by centrifugation. Culture supernatants were sequentially filtered through 0.65- and 0.45-μm filters to remove capsule and other cellular debris and centrifuged for 3 h at 40,000 × *g*. The initial pellet containing outer membrane vesicles was resuspended in ~5 ml of supernatant and centrifuged for an additional 3 h at 40,000 × *g* to further remove debris. The final OMV pellet was resuspended to a final volume of 1 ml in sterile PBS, total protein content was determined by the Bradford assay (Coomassie Plus; Thermo Pierce), and proteins were visualized by 10% SDS-PAGE gels stained with Sypro Ruby Red protein stain (Molecular Probes). The lipopolysaccharide (LPS) content was determined using the Purpald assay as adapted by Velkov et al. (23) with a standard curve created using purified *Klebsiella pneumoniae* LPS from Sigma.

Relative outer membrane vesicle quantification. Relative OMV production was assessed by a previously described phospholipid assay (24). Briefly, cell-free supernatants were collected by centrifugation at 10,000 × *g* and filtered through a 0.45-μm membrane. Vesicles were then pelleted by high-speed centrifugation (40,000 × *g* for 3 h), washed, and resuspended in MV buffer (50 mM Tris, 5 mM NaCl, 1 mM $MgSO_4$, pH 7.4). An equal volume of chloroform was added, and the vesicles were centrifuged at 6,000 × *g* for 5 min at room temperature (RT). The bottom, organic layer was then collected and treated with an equal volume of ammonium ferrothiocyanate solution (27.03 g/liter $FeCl_36H_2O$, 30.4 g/liter NH_4SCN). The sample was centrifuged again; the bottom layer was collected and dried under nitrogen gas. Dried samples were resuspended in 1 ml of chloroform, and the absorbance at 470 nm was measured. Absorbance values were normalized to the optical density at 600 nm (OD_{600}) of the originating cultures.

Outer membrane protein isolation. Cell fractionation was adapted from a previously described protocol (25). Briefly, cell pellets from 100 ml of mid-late-log-phase cultures without antibiotics were resuspended in a Tris-sucrose solution (20 mM Tris, 20% sucrose [wt/vol], pH 8.0) and then lysed with lysozyme and 0.1 M EDTA. The mixture was incubated on ice, and 0.5 M $MgCl_2$ was added to the lysate. The lysate was centrifuged at 12,000 × *g* for 20 min, supernatants containing periplasmic proteins were removed, and pellets containing the membrane fractions were resuspended in ice-cold Tris (10 mM, pH 8.0). Membrane fractions were sonicated on ice, cellular debris was pelleted (9,000 × *g*, 5 min), and the supernatant containing membranes was then further centrifuged (40,000 × *g*, 60 min) to remove cytoplasmic proteins. The membrane fractions within the pellet were washed with ice-cold Tris, resuspended in distilled water (DI H_2O), and stored at −80°C. Membrane fractions were treated with 1.2 ml of Sarkosyl solution (1.7% Sarkosyl [wt/vol], 11 mM Tris) for 20 min and centrifuged at 40,000 × *g* for 90 min to separate the inner and outer membrane fractions. The pellet containing only the outer membrane fraction was washed in ice-cold Tris and dialyzed overnight in PBS at 4°C. Protein concentration was determined by the Bradford assay (Coomassie Plus; Thermo Scientific).

Quantification of OmpA protein in OMVs. Bacterial strains were grown in 250 ml LB broth for 12 h with cephalothin. OMVs were purified as described above and resuspended in sterile PBS. One microgram of total protein from each strain was then separated and visualized via 12% SDS-PAGE with Sypro Ruby Red staining. ImageJ was used to determine the density of the OmpA band in each strain.

Tissue culture. RAW264.7 mouse macrophage cells were grown in RPMI 1640 medium with 10% fetal bovine sera and penicillin-streptomycin-amphotericin B (Fungizone) at 37°C with 5% CO_2. When confluent, cells were harvested by scraping and seeded into 96-well plates at a concentration of 1×10^5 cells/well. Cells were incubated for 3 h with 0.1 ng of purified vesicles or outer membrane proteins as determined by total protein and then harvested for quantitative PCR (qPCR) assay or enzyme-linked immunosorbent assay (ELISA). Experiments were performed using two separately purified batches of OMVs from each strain. Each experiment utilized ≤4 replicate wells per treatment, and each experiment was performed at least twice.

qPCR of macrophage responses. Total RNA was harvested from cultured macrophages using the Qiagen RNeasy kit with DNase treatment. One microgram of total RNA was reverse transcribed using oligo(dT) primers and Protoscript II reverse transcriptase (New England BioLabs) and column purified. One microliter of purified cDNA was used as the template for each reaction mixture. Real-time PCR was performed on an Eppendorf Mastercycler Realplex 2 instrument using LuminoCt SYBR green (Sigma). The primers used are as previously described and listed in Table 1 (26). Gene expression was normalized to actin production in each sample, and change in expression was determined using the $\Delta\Delta C_T$ method (where C_T is threshold cycle) compared to untreated macrophage transcript levels.

TABLE 1 RT-PCR primers used in this study

Protein encoded by murine gene	Primer sequence (5′→3′)	
	Forward	Reverse
MIP-2	TCCATGAAAGCCATCCGACTGCAT	ACATCCCACCCACACAGTGAAAGA
TNF-α	ACCACTCTCCCTTTGCAGAACTCA	TCTCATGCACCACCATCAAGGACT
IL-1b	TGCTTGTGAGGTGCTGATGTACCA	TGGAGAGTGTGGATCCCAAGCAAT
GM-CSF	AGCAGCAGTCTGAGAAGCTGGATT	ACTCCGGAAACGGACTGTGAAACA
Actin	AGAGGGAAATCGTGCGTGAC	CAATAGTGATGACCTGGCCGT

ELISA. Cytokine protein secretion (macrophage inflammatory protein 2 [MIP-2], GM-CSF [granulocyte-macrophage colony-stimulating factor], interleukin-1β [IL-1β], and tumor necrosis factor alpha [TNF-α]) in macrophage culture supernatant was quantified by ELISA (R&D Systems/BD) according to the manufacturer's instructions.

Statistical analysis. All experiments were performed with ≥4 samples and performed at least twice. Statistical comparisons between groups were performed using single-factor analysis of variance (ANOVA), followed by Tukey's *post hoc* test using XLStat software. The statistical significance was set at P values of ≤0.05.

RESULTS

Porin loss does not alter bacterial growth, cellular integrity, or OMV yield. In order to investigate the impact of porin loss on OMVs, we utilized a collection of clonally related ESBL clinical isolates of *K. pneumoniae* that differentially express OmpK35 and OmpK36. These isolates were collected as part of an outbreak of ESBL-producing *K. pneumoniae* strains involving 150 patients. The two strains (CSUB10S and CSUB10R) were found by pulsed-field gel electrophoresis (PFGE) to be clonally related both to each other and to a strain common to all isolates of the epidemic (8, 21). We therefore chose to study these strains as examples of the porin changes naturally selected for in the clinical setting. Strain CSUB10S expresses only OmpK36, while strain CSUB10R does not express either OmpK35 or OmpK36. In order to be able to differentiate between the effects of changing a single porin versus larger-scale changes that may occur in the clinic, we further transformed these strains with plasmids carrying either *ompK35* or *ompK36*. This resulted in the creation of strains expressing only OmpK35 (CSUB10R-pSHA16K) or OmpK36 (CSUB10R-pSHA21), all in the same genetic background of CSUB10R. We further transformed CSUB10S to create a strain that expressed both porins (CSUB10S-pSHA16K). Using these five strains, we have a panel of porin loss strains derived from clinical isolates that exhibit differential expression of OmpK35 and OmpK36 both separately and in combination.

To evaluate the effects of these changes in porin expression on bacterial physiology, we compared the rates of growth and levels of cellular integrity of these strains. All strains were grown in the presence of cephalothin antibiotic to provide the appropriate antibiotic stress. As seen in Fig. 1A, neither porin loss nor transformation with a porin containing plasmid significantly altered the growth rates of these strains. Strains transformed with a plasmid were maintained in the presence of kanamycin, and the growth of these strains was not altered by the presence or absence of this second antibiotic (data not shown).

Altered outer membrane porin composition may impact membrane integrity, leading to increased OMV production or even cellular lysis. Therefore, we tested membrane permeability via both disc diffusion assay and cellular lysis and leakage by Western blotting for the cytoplasmic RecA protein in concentrated culture supernatants. Disc diffusion assays revealed all five strains to be completely resistant to 100 μg SDS and to have equivalent susceptibilities to novobiocin (80 μg per disc; diffusion halo, 22 ± 1 mm for all strains). As seen in Fig. 1B, no significant cellular lysis was detected in bacterial strains with any combination of porin expression.

Antibiotic exposure, membrane stress, and membrane instability have all been shown to stimulate increased OMV production (27–29). We therefore utilized a phospholipid-based assay (24) to determine if porin loss impacted the yield of outer membrane vesicles. All standard assays of OMV production do so indirectly by measurement of either lipid or specific protein content (30). We utilized a lipid-based assay to avoid the confounding factors of changes to the protein composition. Our data (Fig. 1C) indicate that porin loss by itself did not significantly alter the yield of outer membrane vesicles. A small, but not statistically significant, increase in OMV production was observed in both parent strains CSUB10S and CSUB10R. These data together indicate that porin loss in an ESBL background does not cause the type of stress on the integrity of the bacterial envelope that would result in significantly increased outer membrane vesicle production.

Porin loss does impact the protein composition of outer membrane vesicles. Porins are known to be major protein components of outer membrane vesicles from many bacterial species. Therefore, we predicted that porin loss would be reflected in the protein composition of OMVs. As seen in Fig. 2, purified vesicles exhibit the characteristic porin expression pattern of the source bacterial cell. OmpA, a major structural component of the outer membrane, is clearly visible in all samples. The ~46-kDa band is the correct size to be LamB, an alternative porin that has been shown to be upregulated in these porin loss strains (31). Other bands found in some but not all samples may represent other alternative porins, such as OmpK26 (14). These data indicate that porin loss is reflected in the OMV protein composition and is accompanied by changes in expression of other membrane components that may be included in secreted vesicles.

OmpA is a major structural component of both the outer membrane and secreted vesicles and is thought to play a role in the formation and active secretion of OMVs (32). OmpA has also been demonstrated to be a significant virulence factor of *Klebsiella* (33). While OmpA is clearly present in the OMVs of all four strains, we investigated if there were significant differences in the amounts of OmpA incorporated into secreted vesicles. Relative OmpA protein content was determined by densitometry of the OmpA band in OMV preparations per standard total protein content. As seen in Fig. 3, OmpA protein content in OMVs is significantly increased in strain CSUB10S, which naturally expresses only OmpK36. In all other strains, OmpA levels are equivalent.

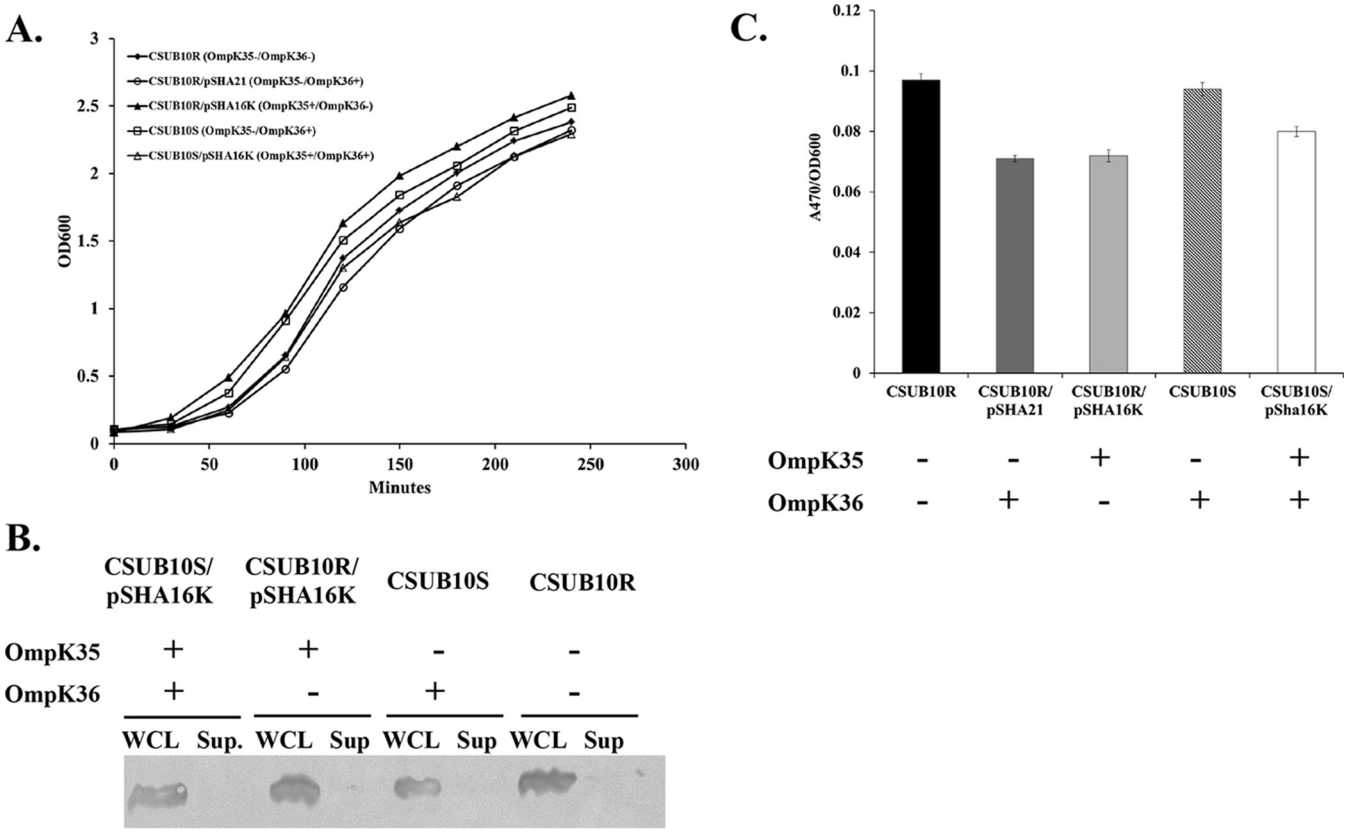

FIG 1 Porin loss does not significantly alter growth, cellular integrity, or the rate of OMV production. (A) Growth curves of *Klebsiella pneumoniae* clonally related isolates with distinct expression patterns of Ompk35 and OmpK36. (B) Western blot of cytoplasmic RecA in whole-cell lysate (WCL) and concentrated culture supernatant (Sup.) to detect cell lysis or leakage due to changes in membrane composition. (C) Outer membrane vesicle production as determined by total phospholipid assay. Culture supernatant phospholipid content was assayed and normalized to the culture CFU. $n = 3$. Bars show means and standard deviations from the means.

These data indicate that there may be a larger shift in outer membrane protein expression and packaging of protein into secreted OMVs that is more complex than just the single porin expression that occurred as clinical isolates CSUB10S and CSUB10R evolved under antibiotic pressure.

Given these changes in protein composition, we then hypothesized that these strains may also exhibit altered levels of lipids such as LPS, which are known to contribute to bacterial virulence and inflammation. We therefore determined the ratio of LPS to protein content in OMVs from each strain by the Purpald assay. Samples of purified OMVs were loaded with a standard protein content, as determined by the Bradford assay, and LPS concentrations were determined by a standard curve to purified *Klebsiella pneumoniae* LPS. OMVs from all strains were found to have the same ratio of LPS to protein content of 10:1 (data not shown). These data indicate that a porin loss phenotype results in significant changes to the protein composition of secreted vesicles but does not alter the quantity of LPS in purified vesicles.

Loss of multiple porins decreases inflammatory responses to OMVs. We then investigated how changes in porin expression impacted macrophage cytokine responses. As assayed by real-time PCR (Fig. 4A), loss of either OmpK35 or OmpK36 singly had little effect on cytokine responses. However, loss of both porins together significantly decreased transcription of all four cytokines assayed. Assaying cytokine protein secretion revealed a more complex pattern, indicating that posttranscriptional controls of cytokine secretion may differentiate responses to OMVs from that of whole bacterial cells. IL-1β secretion is dependent on activation of the inflammasome signal transduction pathway, which results in caspase-1 cleavage of the pro-IL-1β protein into the active, secreted form. In our experiments, IL-1β secretion was not observed (Fig. 4B), even with exposure to increased doses of OMVs (data not shown). These data indicate that OMVs from *Klebsiella* do not contain sufficient signals to trigger active IL-1β secretion.

Secretion of MIP-2, a neutrophil chemotactic factor, was highly sensitive to porin composition (Fig. 4B). OMVs from CSUB10R, containing neither OmpK35 nor OMpK36, triggered very low levels of MIP-2 secretion. Restoration of either porin in this isogenic background restored a significant level of MIP-2 production. However, OMVs from strain CSUB10S, which naturally expresses OmpK36, triggered even higher levels of MIP-2 secretion. This high level was maintained even when expression of OmpK35 was added back via a plasmid. These data indicate that MIP-2 secretion is responsive to both the specific porin content in strain CSUB10R and other changes in composition, such as in OmpA content, that occurred during the evolution in the clinic of strains CSUB10R and CSUB10S.

The importance of OmpK35 and OmpK36 in innate responses

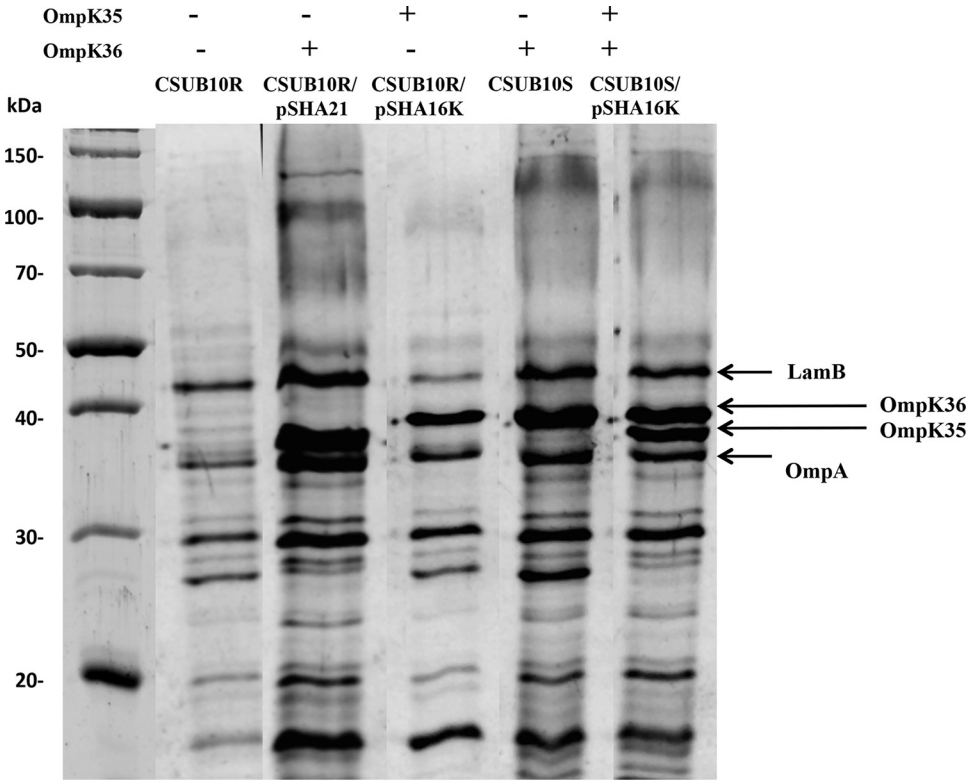

FIG 2 Strains of *Klebsiella pneumoniae* exhibit porin loss in the protein composition of secreted outer membrane vesicles. Gels (10% SDS-PAGE) of purified outer membrane vesicles from strains CSUB10R (OmpK35−/OmpK36−), CSUB10R/pSHA21 (OmpK35−/OmpK36+), CSUB10R/pSHA16K (OmpK35+/OmpK36−), CSUB10S (OmpK35−/OmpK36+), and CSUB10S/pSHA16K (OmpK35+/OmpK36+). Arrows indicate the positions of OmpK35, OmpK36, OmpA, and LamB.

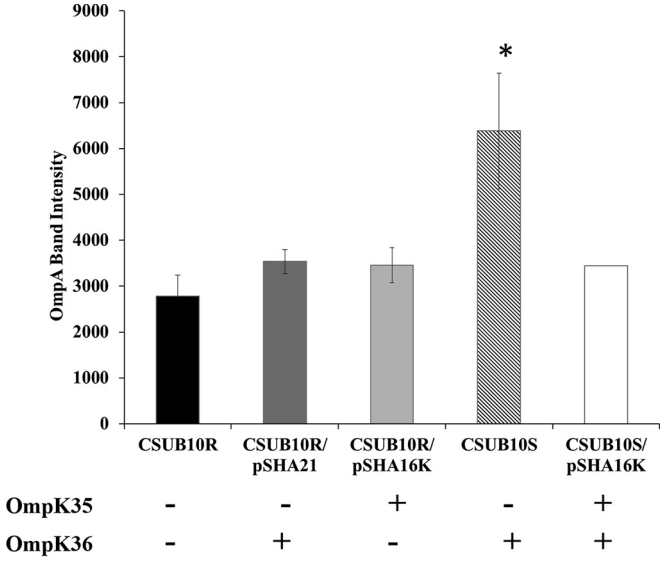

FIG 3 Effects of OmpK35 and OmpK36 expression on OmpA inclusion in outer membrane vesicles. The relative OmpA content in each strain was determined by densitometry of the OmpA band in 12% SDS-PAGE gels. OMV samples were purified in a standard volume, and the equivalent protein content from each sample was separated by SDS-PAGE. $n = 3$. Bars show means and standard deviations. Significance between all strains was determined by ANOVA and Tukey's *post hoc* test. *, $P < 0.05$, $\alpha = 0.05$.

is further seen by the pattern of TNF-α secretion. Loss of both porins was required to significantly alter TNF-α production, as the presence of a single porin in either genetic background resulted in full levels of secretion. These data may indicate that recognition of a common porin structure, such as a beta-barrel motif, may directly trigger production of TNF-α. In contrast, loss of OmpK36 alone in the CSUB10R background significantly increased GM-CSF production above all other treatments. In fact, the lowest level of GM-CSF secretion was in response to vesicles containing both porins.

The protein composition of OMVs is known to differ from that of the source outer membrane. The contents of OMVs are known to be selectively packaged, enriching in molecules that may impact inflammation (20, 34). Therefore, we tested the proinflammatory cytokine responses of macrophages exposed to enriched total outer membrane from strains expressing neither porin, either porin, or both porins to determine if the responses were similar. As seen in Fig. 4C, exposure to equivalent doses of outer membrane proteins resulted in low levels of MIP-2 and GM-CSF, equivalent across all four strains. Secretion of either TNF-α or IL-1β was not detected at this dose. These data indicate that naturally secreted OMVs are more potent stimulators of cytokine production than the purified source outer membrane. Enriched membrane fractions have a disrupted three-dimensional (3-D) configuration and possible washing out of immunostimulatory lipids such as LPS. These factors may in part explain the highly potent response to intact secreted vesicles.

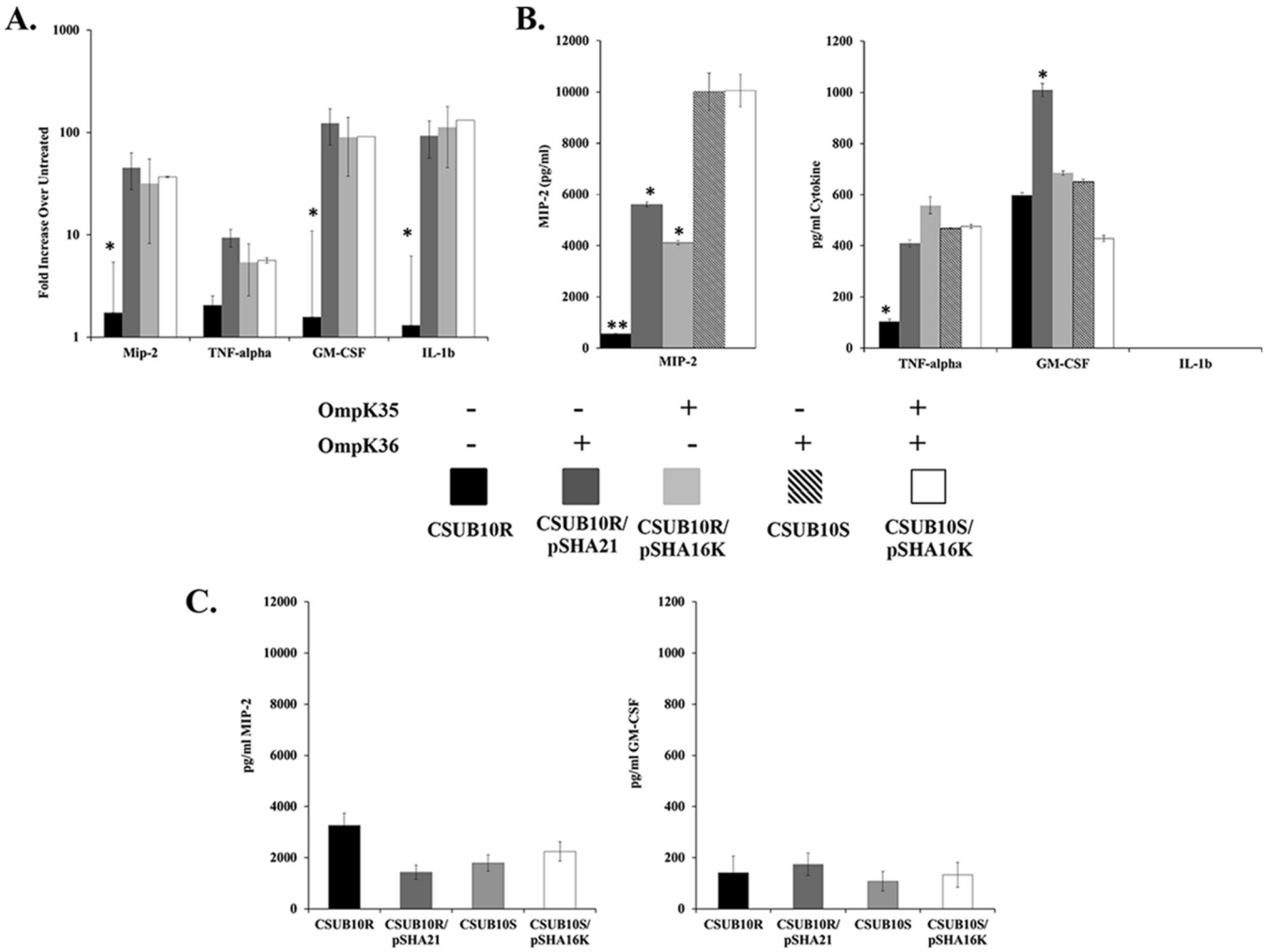

FIG 4 Porin composition of outer membrane vesicles impacts the macrophage inflammatory response. RAW 264.7 mouse macrophages were incubated for 3 h with 0.1 ng protein content of outer membrane vesicles or enriched outer membranes from each bacterial strain. Cytokine production to OMVs was determined by real-time PCR (A) and ELISA (B). Cytokine secretion in response to the enriched outer membrane fraction was determined by ELISA (C). Bars show standard errors. $n \geq 4$ for each experiment, and each experiment was performed at least twice. Significance from all other groups was determined by ANOVA and Tukey's *post hoc* test. *, $P < 0.05$, $\alpha = 0.05$; **, $P < 0.001$, $\alpha = 0.05$.

DISCUSSION

The goal of the present study was to characterize the impact of antibiotic resistance-associated porin loss on the production, composition, and inflammatory capacity of secreted outer membrane vesicles of *Klebsiella pneumoniae*. We found that the composition of OMVs from ESBL-resistant strains exhibit the specific porin loss of the source strain, as well as other changes in outer membrane protein composition. These alterations in the outer membrane did not impact the OMV yield or overall membrane integrity. OMVs from these strains triggered a potent proinflammatory cytokine response in macrophages that was greater than that of the purified source outer membrane and was impacted by porin expression. Loss of both the OmpK35 and OmpK36 porins resulted in OMVs with a significantly reduced ability to trigger an inflammatory response.

We hypothesized that loss of the porin OmpK35 or OmpK36 would be reflected in OMV composition. These two proteins are homologues to the *Escherichia coli* OmpF and OmpC proteins, respectively, which are both known major components of vesicles from *E. coli* (35). Additionally, we found that OMVs exhibit other changes in protein composition that can be directly associated with loss of these major transport proteins. Alternative porins, such as LamB, OmpK26, and OmpK37, have been reported as upregulated in a number of resistant isolates exhibiting loss of the major porins OmpK36 and OmpK35 (14, 31, 36). The strains used in our study have been documented to express the LamB protein; and expression of this alternative porin is required for loss of OmpK36 expression and concomitant resistance to cefoxitin (31). LamB was likely incorporated into the OMVs from our strains, although notably less prominent in OMVs from the strains lacking only OmpK36 (CSUB10R and CSUB10R/pSHA16k). This may be due to LamB serving an essential cellular function when OmpK36 is missing and therefore being retained within the membrane.

These results indicate that porin loss associated with antibiotic resistance is not simply a one-gene effect but rather may be part of a suite of changes to the overall porin and protein composition of both the outer membrane and secreted vesicles. The pattern of

MIP-2 secretion supports this hypothesis, as restoration of either single porin in the CSUB10R background resulted in moderate MIP-2 secretion, while OMVs from strain CSUB10S, which also contains only OmpK36, were significantly increased. Furthermore, more-subtle changes in protein composition than loss of a single porin may significantly contribute to these differences in MIP-2 production. These data support the idea that the antimicrobial pressures seen in the clinic may trigger much more complex changes to the bacterial outer surface than previously appreciated. Our lab is currently working to characterize how loss of a single porin causes other membrane changes by using isogenic knockouts to develop a more complete understanding of how antibiotic exposure alters the outer membrane.

Outer membrane vesicles have a protein and lipid composition that is similar to that of the bacterial cell envelope but distinctive from that of the source outer membrane. Studies in a number of Gram-negative species have demonstrated that proteins are selectively packaged and concentrated into outer membrane vesicles (16, 20, 37). This enrichment effect is central to OMVs' role in bacterial pathogenesis. The heterogeneous composition of OMVs is known to contain a range of virulence factors, including protein adhesins, toxins, and enzymes, as well as being laden with innate immune-activating pathogen-associated molecular patterns (PAMPS), such as lipopolysaccharides (38). Secreted vesicles have been demonstrated to be highly enriched in and to act as a primary mode of delivery for bacterial toxins (39). Vesicles can also modify host cell functions, such as inhibiting lysosomal fusion with bacterium-laden endosomes, and can act as potent triggers of inflammation (17). Experimental evidence across many Gram-negative species all point to vesicle formation being an essential physiological function of bacteria that can significantly impact the course of an infection and the host response (17, 18).

OMVs are potent stimulators of inflammation, as seen by the significant difference in levels of response between vesicles and the purified membrane fraction. Purified OMVs contain an intact three-dimensional (3-D) structure, allowing LPS and protein components to interact with macrophages in a natural, native conformation. In contrast, enriched outer membrane fractions are a disrupted collection of protein and lipid. Ellis et al. (26) demonstrated the 3-D requirements for this potent response using OMVs from *Pseudomonas aeruginosa*. In that study, we dissected the molecular components of OMVs that triggered macrophage inflammatory responses. These experiments confirmed that the three-dimensional configuration of OMVs results in a significantly more potent response than to purified LPS. We further compared the responses to OMVs from a clinical isolate and a lab strain, showing how different bacterial strains elicit different levels of inflammatory response. Interestingly, the differences seen between *Pseudomonas* OMVs from highly disparate strains were not as significant as the differences seen in this study between highly related strains that exhibit only the major difference of porin loss (40). The data presented here illustrate how intact OMVs and changes to their composition can dramatically impact the inflammatory response.

Loss of both OmpK35 and OmpK36 had the effect of dampening the overall inflammatory response, with lower levels of cytokine secretion in response to these vesicles. This change in inflammatory responses is not due to changes in LPS, as the relative LPS content in OMVs was not altered by changes in porin expression. The patterns of TNF-α and MIP-2 secretion both demonstrate that outer membrane porins are critical elements recognized by macrophages. Changes in expression of only one or two porins can dramatically alter the host response and may be a key factor in triggering inflammatory cytokine production. This may indicate that a common beta-barrel-type motif, distinctive to bacterial porins (41), may be the recognized signal for TNF-α and MIP-2 production.

The data investigating the role of OmpK36 in virulence are contradictory. OmpK36 is the most common porin lost in antibiotic-resistant strains of *K. pneumoniae*. It is a general-purpose transport porin, with a relatively broad pore size that allows for the transport of a variety of chemical components across the outer membrane (42). Additionally, OmpK36 is one of a small number of proteins in *Klebsiella* that have been investigated as potential virulence factors. *In vivo* mouse experiments have indicated that OmpK36 is required for virulence, as strains lacking this porin are more readily cleared by phagocytes (2, 11, 13). This would indicate that porin loss strains would be at a competitive disadvantage and be more likely to be cleared by the host immune system. However, OmpK36 has also been shown to activate the complement cascade (43, 44), indicating that loss of this porin may help to prevent flagging of the bacteria for phagocytic clearance.

Our data indicate that loss of OmpK36 significantly decreases MIP-2 production and that the loss of two porins dramatically dampens the production of multiple proinflammatory cytokines. MIP-2, a major neutrophil chemotactic factor, has been shown to be critical for clearance of *K. pneumoniae* infection. Treatment of mice with anti-MIP-2 antibodies increased lethality to *Klebsiella* infection via decreased polymorphonuclear monocyte (PMN) infiltration by as much as 60% (45). The sensitive MIP-2 response seen in our data lends credence to the idea that OmpK36 is recognized by innate immune receptors and is particularly involved in triggering MIP-2 production.

Strain CSUB10S, which naturally expresses only OmpK36, also exhibited increased levels of OmpA within secreted vesicles. OmpA, like other porins, is a beta-barrel transmembrane protein. However, it also serves to maintain the integrity of the bacterial envelope via a periplasmic domain that stably interacts with the peptidoglycan layer (46, 47). Beyond its structural role, OmpA has also been investigated as a *K. pneumoniae* virulence factor. Purified OmpA protein has been shown to interact with macrophages and dendritic cells to trigger proinflammatory cytokine production via Toll-like receptor 2 and the NF-κB pathway (48, 49, 50). However, experiments utilizing OmpA knockout bacteria indicate that loss of OmpA results in bacteria that are more sensitive to antimicrobial peptides such as polymyxin B, are more susceptible to phagocytosis, and trigger increased inflammatory responses (33).

These apparently contradictory data may be in part explained by OMV production. Loss of OmpA has been demonstrated in multiple bacterial species to be associated with a dramatic increase in OMV yield (51–53). Therefore, OmpA knockout strains are likely secreting large quantities of vesicles laden with LPS and other porins that trigger a more intense inflammatory response. In this context, the increased OmpA content observed in strain CSUB10S, which expresses only OmpK36, may be linked to the dramatically high levels of MIP-2 secretion seen in this strain. OMVs from strain CSUB10R-pSHA21 do not have an elevated OmpA content, also contain only OmpK36, and do not trigger nearly as high levels of MIP-2. In contrast, this strain does trigger

significantly higher levels of GM-CSF than does strain CSUB10S, indicating that neither OmpK36 nor OmpA may be a significant signal for production of this particular cytokine. These data may further indicate that alternative porins, such as LamB, that are upregulated in a double-porin-loss strain, may also play a role in the inflammatory response.

There is increasing evidence that a key element to *K. pneumoniae* pathogenesis is bacterial evasion and minimal generation of a proinflammatory phagocytic response. Mouse models of *Klebsiella* lung infection have revealed that an inflammatory response characterized by infiltration of large numbers of phagocytic cells is required for effective bacterial clearance (54). Depletion studies have confirmed that production of neutrophil chemotactic factors such as MIP-2 and keratinocyte chemoattractant (KC) is essential to an effective immune response (45, 55). Further, exogenous administration of TNF-α increased the rate of clearance and survival from *K. pneumoniae* lung infection (56). These data all point to the ability of *Klebsiella* to evade or dampen the host inflammatory response, such as the low cytokine response to strain CSUB10R, as being a key element to bacterial survival in host tissues.

The molecular component required for immune evasion by *Klebsiella* has long been assumed to be the polysaccharide capsule, which cloaks the bacterial cell and inhibits binding of soluble complement proteins, opsonizing antibodies, and phagocytic cells. Capsule polysaccharides may also be a component within OMVs that contributes to the inflammatory response. More-recent studies have identified other virulence proteins as also being part of an array of anti-inflammatory mechanisms. The O antigen of LPS, siderophore proteins, and components of the pullulanase type 2 secretion system, as well as the capsule, have all been identified as contributing to immune evasion. Noncapsule components have been shown to directly interfere with NF-κB-mediated signaling pathways within macrophages to prevent inflammatory cytokine secretion (11, 57). This direct interference with innate immune signaling pathways requires direct contact between the bacterium and the immune cell, indicating that outer membrane vesicles are not involved.

However, alterations in the porin composition of outer membrane vesicles may dampen the inflammatory potential of these secreted elements, helping to shield a more vulnerable resistant strain from phagocytic clearance. OMVs have been demonstrated to serve a protective function by binding to phages and antimicrobial peptides such as polymyxin B (27). Further, OMVs have been shown to contain β-lactamase enzymes capable of drug inactivation (58, 59) and to serve as a mechanism for horizontal gene transfer, disseminating DNA containing antibiotic resistance genes to other bacterial species (60). These results illustrate that the functions of secreted OMVs are varied and complex and that the ability of OMVs to act as a physical shield from antibiotic penetration must be balanced with their ability to activate an inflammatory immune response.

In summary, the data presented here demonstrate that ESBL strains of *Klebsiella pneumoniae* naturally secrete outer membrane vesicles with a composition that reflects, but is also more complex than, the porin loss phenotype of the source cells. Vesicle production, which is known to increase in response to bacterial stress, is not impacted by the porin loss phenotype. The protein composition of OMVs from these strains reflects the larger number of changes in outer membrane protein expression that accompany porin loss. The macrophage responses to these OMVs were distinctly more potent than to purified outer membrane and demonstrate that porin composition is a key determinant of the intensity of inflammatory cytokine production. It has recently been demonstrated that vaccination with OMVs from *Klebsiella pneumoniae* is capable of generating an effective protective response (61). However, this new study does not consider the impact of alterations to OMV composition such as those exhibited with porin loss. Therefore, any development of the vaccine potential of OMVs must take these clinically driven alterations to the bacteria into account.

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