Defining principles that influence antimicrobial peptide activity against capsulated *Klebsiella pneumoniae*

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The extracellular polysaccharide capsule of *Klebsiella pneumoniae* resists penetration by antimicrobials and protects the bacteria from the innate immune system. Host antimicrobial peptides are inactivated by the capsule as it impedes their penetration to the bacterial membrane. While the capsule sequesters most peptides, a few antimicrobial peptides have been identified that retain activity against encapsulated *K. pneumoniae*, suggesting that this bacterial defense can be overcome. However, it is unclear what factors allow peptides to avoid capsule inhibition. To address this, we created a peptide analog with strong antimicrobial activity toward several *K. pneumoniae* strains from a previously inactive peptide. We characterized the effects of these two peptides on *K. pneumoniae*, along with their physical interactions with *K. pneumoniae* capsule. Both peptides disrupted bacterial cell membranes, but only the active peptide displayed this activity against capsulated *K. pneumoniae*. Unexpectedly, the active peptide showed no decrease in capsule binding, but did lose secondary structure in a capsule-dependent fashion compared with the inactive parent peptide. We found that these characteristics are associated with capsule-peptide aggregation, leading to disruption of the *K. pneumoniae* capsule. Our findings reveal a potential mechanism for disrupting the protective barrier that *K. pneumoniae* uses to avoid the immune system and last-resort antibiotics.

**Results**

Capsule Promotes Differential Sensitivity of Gram-Negative Bacteria to Synthetic Antimicrobial Peptide PepC. From previous screening campaigns (22), we identified a 17-amino acid antimicrobial peptide, PepC, with bactericidal activity against *Escherichia coli* (Fig. 1A and *SI Appendix*, Fig. S1). Further testing showed that PepC had antibacterial activity toward additional gram-negative bacteria, including *Acinetobacter baumannii* strains, but little activity toward clinical MDR and hypermucoviscous *K. pneumoniae* isolates (23) (Fig. 1B and *SI Appendix*, Table S1). We hypothesized that PepC acts through disruption of the bacterial capsule that *K. pneumoniae* uses to avoid our innate immune response and last-resort treatment options.

Here we characterize the synthetic evolution of a peptide inhibited by capsule to a peptide with potent activity against capsulated *K. pneumoniae*. Remarkably, our results indicate that rather than reduced interactions, our active peptide retains binding to capsule and undergoes conformational changes associated with capsule aggregation. We present a model in which peptide-driven sequestration of capsule disrupts this barrier and reduces its ability to protect *K. pneumoniae* against antimicrobial attack. These findings provide insight into improving antimicrobial peptide activity against *K. pneumoniae* and may help strengthen our understanding of the inability of innate host defense peptides to act on capsulated bacteria.

**Significance**

The capsule of *Klebsiella pneumoniae* is composed of extracellular polysaccharides that inhibit the activity of host defense peptides and polymyxins. We generated an active antimicrobial peptide from a previously inactive parental peptide and characterized the interactions of these peptides with *K. pneumoniae* and its capsule. Compared with the inactive parent peptide, we found that our active peptide retained strong binding to capsule but lost structural integrity. These interactions induced capsule aggregation and capsule disruption, a previously undescribed mechanism for promoting antimicrobial activity toward *K. pneumoniae*. This finding may allow further exploitation of this mechanism to destroy the protective capsule that *K. pneumoniae* uses to resist our immune response and antibiotics.

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membrane, because its cationic charge, amphipathic conformation, and predicted α-helical structure are similar to those of other well-studied peptides that disrupt cellular membranes (Fig. 4) (24). To test this hypothesis, we first measured the ability of PepC to promote propidium iodide entry into bacteria, which requires a loss of membrane integrity. Supporting a membrane disruption mechanism and its spectrum of activity, we observed that PepC could promote propidium iodide uptake by *E. coli* and *A. baumannii*, but not by *K. pneumoniae* (SI Appendix, Fig. S2). To complement this analysis, we used DSC3 dye to measure inner membrane depolarization of *E. coli* and found that the membrane depolarized in a dose-dependent manner with increasing concentrations of PepC (SI Appendix, Fig. S2D).

We hypothesized that the variation in PepC activity was unlikely due to differences in bacterial membranes, since the membrane compositions of *E. coli*, *A. baumannii*, and *K. pneumoniae* are similar (25). However, the capsule of *K. pneumoniae* is more robust than other gram-negative capsules and inhibits the activity of host defense peptides (12). Since PepC shares common physicochemical similarities with many host defense peptides, we hypothesized the antimicrobial activity of PepC is also inactivated by capsule. To test this hypothesis, we assayed PepC activity toward *K. pneumoniae* MKP103 and its isogenic capsule-deficient *wza::180T*30 transposon mutant (26). PepC did not show a minimal inhibitory concentration (MIC) against the wild-type strain up to the maximum dose tested (MIC >128 μM) but did inhibit the capsule mutant (MIC 32 μM) (Table 1). Addition of exogenous purified *K. pneumoniae* capsule to the MIC assay restored PepC resistance (MIC >128 μM) to the *K. pneumoniae* capsule mutant (Table 1). Furthermore, addition of *K. pneumoniae* MKP103 capsule extract inhibited PepC antimicrobial activity against *E. coli* (Table 1). These results support our hypothesis that capsule inhibits the ability of PepC to kill *K. pneumoniae*.

Increasing PepC Activity.

The mechanism behind capsule inhibition of host defense peptides, and how to overcome this inhibition, are unclear. As a relatively short synthetic sequence with typical antimicrobial peptide charge (27), PepC provides a simple template for testing how amino acid changes might influence activity toward capsulated *K. pneumoniae*. We aimed to increase the antimicrobial activity of PepC toward *K. pneumoniae* and use the resulting peptide to understand the variations in capsule–peptide interactions between active and inactive peptides with similar global physicochemical properties.

Mutating each position in PepC to all alternative amino acids is not feasible. Capsule has been shown to bind antimicrobial peptides using electrostatic and hydrophobic interactions to inhibit their antimicrobial activity (16, 17). To explore the impact of these interactions on peptide activity, we generated analogs with variations in the basic and hydrophobic amino acid residues. Basic amino acids arginine, lysine, and 2,4-diaminobutyric acid (DAB) have different structures, potentially facilitating differential electrostatic interactions with capsule and anionic lipids on the bacterial surface. When considering hydrophobicity, we hypothesized that the amino acid tryptophan may have an effect on capsule–peptide interactions because of its extensive π-electron system, which allows it to more effectively interact with the interfacial region of the bacterial membrane (28, 29).

Our resulting analogs displayed a range of antimicrobial peptide activity, with several showing enhanced activity toward *K. pneumoniae* (SI Appendix, Table S2). Analogs A6, A12, and A19 had the lowest overall MICs toward *E. coli* and *K. pneumoniae*. Their MICs against *K. pneumoniae* were 2 μM, compared with >128 μM for parent PepC, indicating that *K. pneumoniae* active antimicrobial peptides could be derived from inactive parent sequences. The activity of A6, A12, and A19 also improved against *E. coli*, suggesting these analogs simultaneously increased their ability to broadly act against bacterial membranes and avoid capsule inhibition. However, these two properties are not always connected, as peptide variants like A5 showed enhanced activity against *E. coli* with only a minor change in activity toward *K. pneumoniae* (SI Appendix, Table S2).

Peptides with the most activity toward *K. pneumoniae* (A6, A12, and A19) had increased tryptophan abundance and used lysine or DAB rather than arginine for basic residues. The substitution of tryptophan resulted in increasing peptide activity, with several additional tryptophan-rich analogs (A9, A16, A17, and A18) having improved MICs against *K. pneumoniae*, ranging from 4 μM to 8 μM. Interestingly further exploration of A19, hereafter referred to as PepW, revealed no difference in MIC toward the capsule mutant and only a twofold increase in MIC with the addition of extracted capsule (Table 1). This was in stark contrast to PepC, which showed a marked difference in MIC between wild-type and capsule mutant strains, suggesting that PepW had gained attributes in addition to an increase in MIC. We also observed that PepW induced membrane disruption by propidium iodide uptake of *K. pneumoniae* MKP103 (SI Appendix, Fig. S3) indicating that the peptide is able to bypass the capsule barrier.

**Table 1.** PepC and PepW MICs toward *E. coli*, *K. pneumoniae*, and capsule-deficient *K. pneumoniae* mutant in the absence and presence of exogenous *K. pneumoniae* capsule.

| Bacterial strains | PepC MIC, μM | PepW MIC, μM |
|-------------------|-------------|-------------|
| *K. pneumoniae* MKP103 | >128 | 2 |
| *K. pneumoniae* wza::180T30 capsule mutant | 32 | 2 |
| *K. pneumoniae* wza::180T30 capsule mutant + CPS | >128 | 4 |
| *E. coli* W3110 | 8 | 1 |
| *E. coli* W3110 + CPS | >128 | 2 |

CPS: *K. pneumoniae* MKP103 10 μg mL⁻¹ capsule.

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To further explore the role of tryptophan, we generated analogs of PepW with tryptophan replaced by other aromatic amino acids (SI Appendix, Tables S2 and S3). With phenylalanine substitution, analog PepF displayed a twofold to fourfold decrease in antimicrobial activity against *E. coli* and *K. pneumoniae* compared with PepW. More notably, we found that tyrosine substitution in PepY had little effect on its MIC toward *E. coli* but resulted in lower activity toward *K. pneumoniae*. Collectively, these results support an important role for tryptophan in activity against capsulated *K. pneumoniae* as well as in promoting broad gram-negative antimicrobial activity.

**PepW Retains Its Activity against *K. pneumoniae* in Vivo.** Since the MIC assays were performed in vitro, it was possible that peptide interactions with *K. pneumoniae* could change in vivo and our peptide analogs would lose activity. To address this possibility, we tested the ability of PepW to act on *K. pneumoniae* in vivo. We first determined that PepW showed limited cytotoxicity in cell culture (SI Appendix, Table S4). We then assayed the ability of PepW to prevent infection in a murine model of lethal *K. pneumoniae* peritonitis. We choose PepW because of its potent *K. pneumoniae* activity in vitro and its incorporation of DAB. DAB peptides show reduced proteolysis in vivo (30), thus reducing degradation as a potential confounding variable of PepW in vivo activity. *K. pneumoniae* MKP103 was inoculated using an intraperitoneal (i.p.) injection of ~5 × 10⁵ CFU, followed by an i.p. injection of either PBS or 5 mg kg⁻¹ PepW in PBS. After 24 h, the mice were killed, and organs were harvested for bacterial enumeration (Fig. 2). We saw a significant decrease in bacterial dissemination to the liver, kidneys, heart, and lungs. The PepW-treated mice had 2-log fewer bacteria in each organ assessed. These results indicate that PepW remains active in a complex in vivo environment, and that our results are relevant to understanding peptide–capsule interactions in a host.

**PepW Retains Binding and Loses Structure in the Presence of Capsule.** To begin to understand how the inactive parent PepC could evolve into the active analog PepW, we investigated their binding to capsule. The ability of capsule to bind and sequester antimicrobial peptides has been suggested as a way to protect bacteria from eradication (9). Thus, we anticipated that PepW might show decreased capsule binding. To test this, we incubated PepC and PepW with extracted capsule and passed the sample through a 100-kDa filter to remove capsule-bound peptide while collecting the remaining free peptide, as described previously (16). Interestingly, our analysis on an sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) gel revealed similar binding of PepC and PepW to capsule (Fig. 3A). Both peptides displayed a dose-dependent decrease in band density as the concentration of capsule was increased from 0.5 to 4 µg mL⁻¹, indicating binding to capsule. To quantify these results, we performed band densitometry analysis to determine the percent bound of PepC and PepW (Fig. 3B) (16). This confirmed our qualitative SDS-PAGE results and showed that at each concentration, PepW bound capsule at least as strongly as PepC.

In addition to binding peptides, previous studies revealed that capsule extract from *K. pneumoniae* induced host defense peptide secondary structure, and that this was associated with their loss of antibacterial activity (12). We investigated whether PepC or PepW underwent structural changes following the addition of capsule using circular dichroism (CD) as described previously (12, 16, 31). In buffer alone, both PepC and PepW displayed an α-helical character with negative minima near 208 nm and 222 nm (Fig. 3C and D). Despite their similar length and global amphipathic character, the peptides reacted differently to the addition of capsule. The addition of capsule caused a dose-dependent loss of α-helical structure in PepW but appeared to induce secondary structure in PepC. Thus, PepC and PepW behaved differently to the addition of capsule using circular dichroism (CD) as described previously (12, 16, 31). In buffer alone, both PepC and PepW displayed an α-helical character with negative minima near 208 nm and 222 nm (Fig. 3C and D). Despite their similar length and global amphipathic character, the peptides reacted differently to the addition of capsule. The addition of capsule caused a dose-dependent loss of α-helical structure in PepW but appeared to induce secondary structure in PepC. Thus, PepC and PepW behaved differently to the addition of capsule using circular dichroism (CD) as described previously (12, 16, 31). In buffer alone, both PepC and PepW displayed an α-helical character with negative minima near 208 nm and 222 nm (Fig. 3C and D). Despite their similar length and global amphipathic character, the peptides reacted differently to the addition of capsule.

**Limited and Separate Amino Acids Promote PepC and PepW Interactions with a Model Polysaccharide.** Our results indicate that PepC and PepW interact with capsule with similar affinities but significantly different outcomes for both their structure and their activity. We used native mass spectrometry (32–36) coupled with UV photo-dissociation (UVDP) (37–42) to better understand how these peptides differ in their polysaccharide interactions. UVDP of non-covalent peptide–ligand complexes produces sequence ions that retain the ligand, termed holo ions, as well as ligand-free sequence ions termed apo ions (43). The resulting fragmentation patterns can be used to determine binding sites, essentially localizing residues or regions that interact with the ligand (37–43). In particular, binding sites are localized based on overlap observed in holo-sequence ions containing the N terminus of the peptide and holo-sequence ions containing the C terminus of the peptide. Native bacterial capsule is too heterogeneous to allow successful peptide–polysaccharide analysis by native MS. Therefore, we used stachyose as a simple tetrasaccharide surrogate to enable characterization of PepC and PepW polysaccharide interactions via UVDP-MS (SI Appendix, Fig. S4). Capsule is hypothesized to interact with peptides through both electrostatic and non- electrostatic interactions (16). Stachyose lacks the negative charge present in capsule, but the hydroxyl groups of the stachyose structure are polar, allowing formation of hydrogen bonds with the basic amino acids (44). Therefore, we anticipated that we could still capture important differences in additional types of PepC and PepW polysaccharide interactions. UVDP of PepC–stachyose and PepW–stachyose complexes (3+ charge state) revealed different interactions (Fig. 4). The holo-ion plot for the parental PepC–stachyose complex, illustrated as a function of cleavages along the peptide backbone that result in sequence ions retaining stachyose, revealed interactions driven by three C-terminal amino acid residues arginine 12, tyrosine 13, and arginine 14 (Fig. 4A). This finding aligns with our understanding that basic and aromatic residues of antimicrobial peptides promote interactions with the polysaccharides in capsule (12). Comparatively, analysis of the PepW–stachyose complexes indicated that PepW interacted with amino acids shifted toward the center of the peptide at positions Dab 9, leucine 10,
and leucine 11 (Fig. 4B). In addition, the C-terminal single tryptophan of PepW revealed a fourth interaction with stachyose, but there was no evidence for interactions directly involving the three N-terminal tryptophan residues. These results indicate that while both PepC and PepW share a similar size and amphipathic character, the site-specific variations in their amino acids strongly influence their interactions with this model polysaccharide.

**Stachyose Induces Opposite Structural Changes in PepC and PepW.** To complement the structural changes observed by CD, we assessed the variations in UVPD fragmentation patterns between peptides and extracted capsule. Samples were filtered to remove the capsule and bound peptide fraction. Assays were performed in biological triplicate. A representative image is shown. (B) Densitometry analysis of A to determine the percent peptide bound for PepC and PepW to extracted capsule. Results are from triplicate measurements, with error shown as ± SEM. (C and D) CD profiles of PepC (C) and PepW (D) in the presence of increasing concentrations of extracted capsule.
complexed with stachyose vs. the peptides alone. These variations are displayed as difference plots in Fig. 4 C and D, where negative values represent the suppression of backbone cleavages of the peptide-stachyose complexes relative to the free peptides. This outcome is indicative of an increase in order or secondary structure of the peptide caused by greater networks of noncovalent interactions that mitigate the separation and release of detectable fragment ions. In essence, the difference plots indicate whether each peptide becomes more or less structured in the presence of the surrogate polysaccharide and serve as a secondary assay to validate our previous results using CD. In the presence of capsule, PepC displayed significant suppression of fragmentation along the entire peptide sequence, with even more substantial suppression toward the N and C termini (Fig. 4C). In contrast, peptide analog PepW displayed relatively little change in fragmentation when bound to the polysaccharide (Fig. 4D). PepW even displayed slightly positive values toward the C terminal, indicating an increase in fragmentation, suggestive of a decrease in secondary structure. These mass spectrometry results complement our CD findings suggesting that PepW loses structure in the presence of capsule polysaccharides, while PepC gains structure.

**PepW Induces Capsule Condensation and Disruption.** Our results indicate that the active peptide PepW bound capsule as well as its inactive parent PepC but lost structure when in complex with capsule. To rationalize how this activity could lead to increased antibacterial activity, we hypothesize that the secondary structural change that PepW adopts when binding polysaccharides may cause capsule aggregation and subsequent capsule disruption. A disrupted capsule barrier may then enable the remaining antimicrobial peptides to reach and act on the bacterial membrane. When testing the peptides, we observed that the addition of high concentrations of capsule caused the PepW solution to become turbid while the PepC solution remained clear. This result suggested that PepW was aggregating with the capsule. To test this, we centrifuged solutions of PepC and PepW with and without 160 μg mL−1 of capsule to observe pelleting. We also tested additional peptides that are active (PepK and PepF) or inactive (PepY) against capsule-bound *K. pneumoniae* (SI Appendix, Tables S2 and S3). The active peptide (PepW, PepK, and PepF) solutions produced a large pellet, while PepC produced a small pellet and PepY did not produce a visible pellet (Fig. 5A). Quantification of the capsule present in the aggregates at the bottom of each tube confirmed that significantly more capsule was present in the active peptide aggregates than in the inactive peptide aggregates (SI Appendix, Fig. S5).

We wanted to determine whether we could observe any changes in the bacterial capsule of live cells to complement our capsule aggregation finding. We first used Percoll gradient analysis, which has been used to study the extent of encapsulation for several bacteria, including *K. pneumoniae* (45–48). Under our conditions, untreated *K. pneumoniae* MKP103 formed a line evenly at the 35% line of the Percoll gradient (SI Appendix, Fig. S6). Treatment with PepC produced the same result. Interestingly, we found that a 15-min treatment with PepW induced capsule removal, as demonstrated by turbidity above the line that formed at the 35% layer of the Percoll gradient. No loss of bacterial viability was identified under these conditions (SI Appendix, Table S5). These results indicate that PepW rapidly affects the capsule network, leading to aggregation and disruption of the *K. pneumoniae* capsule.

Finally, we used microscopy to observe changes in *K. pneumoniae* capsule caused by PepW. To facilitate observations in changes to capsule structure, we used a hypermucoviscous K2 serotype isolate (23). Following treatment with buffer, PepC, or PepW, *K. pneumoniae* cells were stained using an India ink negative staining technique to observe capsule surrounding bacteria. A robust capsule was observed around *K. pneumoniae* in buffer alone or treated with PepC (SI Appendix, Fig. S7 A and B), but this capsule layer was greatly diminished following PepW treatment (SI Appendix, Fig. S7C). More detailed images using transmission electron microscopy (TEM) showed a similar result. The negative staining of the control revealed the capsule as a halo around the cell with visible fimbriae (Fig. 5 B and D). Conversely, treatment with PepW showed a disrupted capsule layer (Fig. 5 C and E). Furthermore, we observed areas of minimal capsule associated with membrane distortion and blebbing. These intriguing images validate that PepW disrupts the bacterial membrane and indicate that disruption is often near areas of minimal capsule. These microscopy results support the observations from our in vitro capsule aggregation and Percoll gradient experiments, indicating that PepW is able to disrupt the capsule layer and gain access to the *K. pneumoniae* cell surface to elicit its antibacterial action.

**Discussion**

MDR *K. pneumoniae* is a major concern for human health due to the rapid decline in effective treatments (5, 6, 8, 9, 49).
The capsule of *K. pneumoniae* is known to inhibit host antimicrobial peptide activity and complement lysis by acting as a penetration barrier (8). Although the capsule inhibits the activity of host antimicrobial peptides, there have been synthetic peptides identified with potent activity toward this species (50–52). However, no studies have aimed to determine how *K. pneumoniae* active peptides are superior to host antimicrobial peptides in their ability to penetrate the bacterial capsule.

Previous work focusing on host defense peptides indicated that capsule sequesters the peptides to prevent penetration to the target bacterial membrane (16, 17). Therefore, we made amino acid changes in an attempt to avoid this sequestration. Although the positive charges of peptides have been shown to interact with anionic polysaccharides, the peptide–polysaccharide interactions are more complex and include other interactions, such as van der Waals and hydrophobic interactions (18). Indeed, studies have shown that extracellular polysaccharides can adopt conformations with hydrophobic pockets, and that these pockets are important for capsule binding to antimicrobial peptides (16, 17, 53). While a complete analysis of all amino acid substitutions in PepC was not possible, our selected modifications offer some insight into the importance of basic and hydrophobic amino acids for antimicrobial activity toward capsulated *K. pneumoniae*. While we studied the effects of a variety of basic amino acids, we focused on substitution of tryptophan for leucine and isoleucine to introduce the chemical complexity this amino acid has to offer. Tryptophan has a significant quadrupole moment resulting from the negatively charged electron clouds of the aromatic ring allowing it to form hydrogen bonds with polar interfaces (28). However, we hypothesized that in the presence of basic amino acids, tryptophan might form intramolecular interactions and decrease the number of amino acids capable of hydrogen bonding (54), resulting in a decrease in binding so that the peptide can pass the capsule. However, we found this was not the case for PepW, and that a decrease in capsule binding does not appear to explain its increased activity.

The binding of PepW to capsule should increase its α-helical structure based on previous findings with host defense peptides (12, 16, 17), but this is not what we observed. PepW lost structure with increasing concentrations of extracted capsule, indicating potential peptide aggregation (55). Foschiatti et al. (31) described aggregate formation between cathelicidin peptides and bacterial exopolysaccharides as an explanation for the loss of peptide activity. Considering that both hydrophobic and basic amino acid residues have been shown to increase binding to capsule polysaccharides (18), and that tryptophan is a hydrophobic amino acid able to participate in hydrogen bonding (28), its addition to a peptide may increase both the hydrophobic and the polar interactions with capsule. These binding interactions may facilitate unfolding and cause capsule-peptide aggregation, leading to disruption of the capsule.

Based on our microscopy imaging, we propose that PepW binding to capsule and the subsequent structure loss leads to capsule-peptide aggregation that distorts or removes the capsule, allowing the remaining free peptide to disrupt the bacterial membrane (Fig. 6). This mechanism for antimicrobial peptide activity toward capsulated *K. pneumoniae* offers a ray of light in the fight for effective therapeutics toward this extremely resistant species. Although tryptophan residues play an important role in the evolution of PepW antimicrobial activity, we believe that it is the general physiochemical properties, not a single amino acid residue, that creates this effect. Unraveling the physiochemical properties that allow for this new antimicrobial peptide mechanism would be an important step toward understanding how to destroy the protective layer that *K. pneumoniae* uses to avoid the innate immune response, and this is a future focus of experimentation in our laboratory.

**Materials and Methods**

Detailed information on bacterial strains, growth conditions, bacterial inhibition assays, peptides, eukaryotic cytotoxicity, in vivo testing, membrane disruption assays, capsule extractions and quantifications, CD, capsule binding analysis, Percoll gradients, native mass spectrometry, and microscopy imaging is provided in the *SI Appendix*. Bacterial strains are listed in *SI Appendix*, Table S6.

**Data Availability.** All study data are included in the main text and *SI Appendix*.

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