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Soluble LPS May be a More Potent Competitive Inhibitor to Polymyxin B Than *Escherichia coli* O9a:K30 Capsular Polysaccharides

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Escherichia coli **expresses an extracellular layer of capsular polysaccharide (CPS) that confers protection from environmental stressors such as desiccation and antibiotics. Recent research has suggested a correlation between CPS and resistance to cationic antimicrobial peptides such as Polymyxin B (PmB). It is suggested that CPS acts as a binding target for PmB which prevents interaction with the bacterial surface and hence killing. However, in this model the role of lipopolysaccharide (LPS), the target molecule of PmB, has yet to be assessed. It is known that PmB directly interacts with LPS and inactivates the endotoxin. Similarly, LPS has been shown to interact with PmB. In our study, we investigated the degree to which LPS and CPS neutralize activity of PmB. CPS was extracted and quantified from E69 and the isogenic CPS deficient *E. coli* strain CWG655Δ[wza-wzb-wzcK30]. The degree to which soluble CPS can confer additional resistance to PmB was assessed by a minimum inhibitory concentration assay in liquid broth culture. Our data suggest that soluble LPS neutralizes the bactericidal properties of PmB while soluble CPS may not. LPS may be a more potent inhibitor of PmB than CPS.**

The bacterial capsule, is an extracellular layer of capsular polysaccharide (CPS) that envelopes many species of the *Enterobacteriaceae* family such as *Escherichia coli* (1). CPS has been shown to be released into the surrounding environment, leading to the formation of a slime layer, an unorganized layer of extracellular material which protect the bacteria cells from environmental dangers such as antibiotics and desiccation (2, 3).

E. coli produces a range of cell surface polysaccharides (1). At 37°C the two major surface polysaccharides are lipopolysaccharide (LPS) and CPS (1). Both forms are serotype-specific and are distinguished by the presence of either O-antigen or K-antigen, respectively (4). In particular, the 70 serogroups of CPS K-antigen are further organized into 4 distinct classes based on their method of synthesis and expression (4). *E. coli* O9:K30 expresses Group 1 Capsule with its biosynthesis and surface expression genes housed in the *cps* locus (5). The modulated secretion of CPS upon environmental cues is noteworthy, as its primary function is to resist phagocytosis and enhance adherence (6). Such virulence determinants are essential for proper survival and successful colonization in a host (4).

CPS is a polymer of the repeated subunits of nucleotide-diphospho-sugar (4). Upon synthesis in the cytoplasm, the subunits are transferred onto carrier lipids and flipped to the periplasmic face through the action of glycosyltransferase Wzx (7). On the periplasmic side, Wzy polymerizes these repeated units to produce capsular K-antigen (8). This polymerization has been suggested to be driven and regulated by Wzc tyrosine autokinase (9,10). Little is known about the transport mechanism of CPS through the periplasm. However once the CPS molecule has reached the outer membrane, Wza translocates the molecule to the extracellular surface (10).

The unique and complex mechanisms involved in bacterial membrane stability has, in recent years, led to the development of antibiotics which disrupt outer membrane integrity. For example, Polymyxins have recently re-emerged worldwide as a last-resort treatment to infections caused by multidrug-resistant gram-negative bacteria (11). Polymyxin B (PmB) is a cationic antimicrobial peptide (AP) that exerts it effects by binding to the lipid A component of LPS and competitively displacing the calcium and magnesium bridges that stabilize the LPS (12). This disruptive effect leads to permeability changes in the outer membrane that ultimately result in cell death. Commonly reported resistance mechanisms to polymyxins include the use of efflux pumps, as well as covalent modifications to the lipid A moiety of LPS that decrease its net negative charge and thereby reduce its binding to polymyxins (11).

In recent years, however, researchers have characterized novel mechanisms of resistance to APs that involve the bacterial capsular polysaccharides (13, 14). Campos *et al.* (2004) observed a significant correlation between the amount of CPS expressed by *K. pneumoniae* and relative survival. In addition, the CPS-deficient mutant bound more polymyxin B than the wild-type strain. The researchers hypothesized that CPS plays a direct role in resistance to APs by acting as a shield and reducing their interactions with the outer membrane (13). Follow-up research, however, suggests that cell-free anionic CPSs released from the cell surface traps PmB by acting as a decoy binding target, presumably via electrostatic interactions between cationic PmB and anionic CPS (14). This reduces the quantity of drug that reaches the cell surface and increases PmB resistance. The researchers reported that purified CPSs from *K. pneumoniae* K2 increased the minimum inhibitory concentration (MIC) of PmB for unencapsulated *K. pneumoniae* (14). Furthermore, PmB was observed to trigger the release of CPSs from the bacterial surface, which in turn, reduced the bactericidal activity of PmB. As of now, the role of CPS in mediating polymyxin resistance remains under debate.

The Group I capsules of *E. coli* resemble the capsules of *Klebsiella* strains in terms of structure, genetics, and patterns of expression (15). The Group I capsule clusters from *E. coli* and *Klebsiella* are organized with a conserved block of translocation-surface assembly genes (15). In addition, there are marked structural similarities between Group I K antigens in *E. coli* and *K. pneumoniae* (16, 17). While previous research on CPS-mediated bacterial resistance to APs has centred on *K. pneumoniae*, we explored the role of CPS as a PmB-neutralizing agent on *E. coli* by comparing the effects of CPS extract from the culture supernatants of E. coli E69 cells (serotype O9a:K30:H12) and E. coli CWG655 cells (13; 14). Given the similarities between *E. coli* and *K. pneumoniae* Group I CPS, we hypothesized that the *E. coli* Group I CPS will have an analogous role in the mediation of PmB resistance. That is, soluble *E. coli* Group I CPS confers resistance to polymyxin B by acting as a competitive binding target. To evaluate the effectiveness of *E. coli* Group I CPS as a PmB-neutralizing agent, we performed a PmB minimum inhibitory concentration (MIC) assay on *E. coli* E69 and CWG655 supplemented with CPS at a range of concentrations. Because LPS is also known to interact with and potentially neutralize PmB (18), we compared LPS alongside our extracted CPS. Contrary to our hypothesis, our data suggests that free soluble CPS has minimal, if any effect in neutralizing PmB. Instead, soluble LPS appears to act as the major contributor to neutralizing PmB.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *Escherichia coli* strain E69 was provided by the Whitfield lab at the University of Guelph; strain CWG655 [CWG258 with (wza-wzb-wzc)K30::aphA3; Km^r Sp^r] was given by the Department of Microbiology and Immunology at the University of British Columbia. To confirm the identity of the *E. coli* CWG655 mutant, the strain was grown on kanamycin and spectinomycin (19). For capsular polysaccharide extraction, the strains were grown for 48 hr at 37°C and diluted to 10^6 fold in M9-glucose media before plating 100 µL on M9-glucose agar (20). Cells were grown for 24 hr at 37°C and 48 hr at room temperature before CPS extraction. For minimal inhibitory concentration (MIC) assays, the overnight cultures of strains were grown in Luria-Bertani (LB) medium. For cell supernatant harvesting, *E. coli* strain UB1005 from group 1δ was grown in LB with and without gentamicin at sublethal concentration.

Extraction and purification of K30 capsular polysaccharide. Cell-free CPS was extracted and purified from *E. coli* E69 and CWG655 as described previously (21, 22). Around 200 colonies were grown on M9-glucose agar. Cells were scraped into 30 mL saline and resuspended with a vortex mixer. Cell counts were determined from the suspension by dilution plating. After centrifugation to remove the cells (4,500 x g for 10 min), $MgCl_2$ (final concentration 10 mM), deoxyribonuclease (10 µg/ml), and ribonuclease A (10 µg/mL) were added to the supernatants and the samples were incubated at 37°C for 1 hr. Proteinase K was then added (10 µg/mL), and the samples were incubated at 65°C for 1hr. The enzyme-treated supernatants were dialyzed against 4 L of deionized water for 24 hr at 4°C using tubings with a molecular weight cutoff of 3500 Da with constant, slow stirring. Water was changed at 4 hr, 14 hr, and 20 hr during the dialysis. LPS was then pelleted by ultracentrifugation at 47,000 × g for 16 hr and then at 105,000 × g for 5 hr; however, to fully pellet the LPS, we recommend ultracentrifugation at 105,000 × g for 16 hr. The supernatants were then lyophilized. For MIC assays, CPS was resuspended in deionized water and sterilized with 0.45 µm filters.

Quantification of capsular polysaccharide. Lyophilized cell-free CPS samples were resuspended in 2 mL of water. Uronic acid content of the samples was measured as a proxy of CPS concentrations using a modified carbazole uronic acid assay (23). In test tubes, mix 1 mL of samples or standards with 5 mL of 0.025 M sodium tetraborate in sulfuric acid. The tubes must be closed with marbles or ground glass lids and shaken at first gently, then vigorously with constant cooling. The samples were then heated to 100°C in water bath for 10 min. After cooling, 0.2 mL of 0.125% (w/v) carbazole in absolute ethanol was added, and the samples were heated for another 15 min. Once the samples reached room temperature, the absorbance at 530 nm was measured in quartz cuvettes and compared with a standard curve generated using 4 - 40 µg/ml of glucuronolactone (Alfa Aesar) dissolved in water saturated with sodium benzoate (Sigma). Contamination of chromatographic samples by dust or chlorinated tap water gave a green color found to be due to oxidants. The contamination from oxidants such as sodium nitrite can be corrected by subtracting from the absorbance at 530 nm the absorbance at 920 nm.

Silver stain detection of LPS and protein contaminations in CPS samples. The resuspended CPS samples were allowed to migrate through 12% NuPAGE® Bis-Tris gels for 2 hr with a voltage of 120 volts prior to being visualized using silver stain. Silver staining alone does not visualize CPS but stains LPS and proteins (24, 25). BSA and LPS, serially diluted in deionized water, were used as positive controls. 40 µL of the samples were loaded. Furthermore, the cell culture supernatants of *E. coli* UB1005 in LB-gentamicin and LB were loaded as additional controls. All samples were mixed with 4× NuPAGE® LDS sample buffer prior to loading.

Polymyxin B minimum inhibitory concentration assay. The minimum inhibitory concentration (MIC) assay was performed in duplicates within a standard 96 well plate. Polymyxin B was serially diluted 1:2 across the plate starting at 20 µg/mL final assay concentration, but was adjusted to begin at 1.0 µg/mL in later experiments with CPS due to its potency. Next, prepared sample (LPS or CPS) is added to the wells to a physiologically relevant final concentration (14, 26). LPS was supplied by the Hancock lab (UBC), whereas CPS was purified from previously described methods. Lastly, the cells, diluted to a concentration of 2.0×10^5 was added to each well, wherein each well received 10,000 cells in a final assay volume of 200 µL. After a 16 - 20 hr overnight incubation at 37ºC, turbidity within the wells was assessed by eye as either turbid or clear. The MIC was determined as the minimum concentration of polymyxin B required to limit growth such that no turbidity is observed.

Images and data processing. Photographs of silver stain gels were taken and transformed to grayscale in GIMP. Images were cropped with ImageJ and annotated with Inkscape. Line of best fit for CPS quantification was determined in Excel.

RESULTS

Biochemical analysis shows higher CPS production in strain E69 compared to strain CWG655. CPS was extracted using previously described methods and its concentration was measured using the carbazole sulfuric acid assay. A uronic acid standard curve was prepared using known concentration of glucuronolactone (Fig. S1). CPS samples extracted from strain E69 and strain CWG655 were quantified through the standard curve by converting the

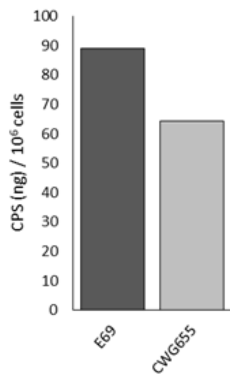

FIG. 1 Extracted CPS per million cells. Unfiltered CPS was quantified by carbazole sulfuric acid assay and normalized with cell counts obtained from dilution plating. WT E69 CPS was quantified as 89.12ng/10^6 cells. Mutant CWG655 CPS was quantified to be 64.31ng/10^6 cells.

absorbance values into the corresponding uronic acid concentrations, which are used as proxies for CPS concentrations (Table S1). We observed that filter-sterilizing the samples led to a loss in CPS. In addition, the concentrations of CPS were higher in extracts of E69 (wild-type) compared to mutant CWG655. CPS produced between the strains were compared by normalizing unfiltered CPS quantifications with cell counts obtained from dilution plating, and expressed as CPS (ng) per million cells (Fig. 1). As expected, extracted free CPS was greater in E69 at 89.12 ng/10^6 cells compared to 64.31 ng/10^6 cells obtained from CWG655, indicating a deficiency in CPS expression within mutant CWG655.

Soluble LPS has an effect in neutralizing polymyxin B mediated inhibition of cell growth. In order to determine if concentrations of LPS produced at normal cell conditions had an effect on polymyxin B activity, and thus influence our measurement of CPS conferred resistance, we first performed an LPS dosing assay. We showed that increasing concentrations of LPS resulted in an increase in the MIC for polymyxin B, in which higher LPS concentrations required higher polymyxin B concentrations to inhibit cell growth (Table 1). Because of a strong LPS effect observed, an ultracentrifugation step to purify the CPS was performed to limit any possibility of LPS interference during the measurement in effect of soluble CPS neutralization of polymyxin B.

Detection of LPS and protein contamination in extracted cell-free CPS using silver stain. Silver staining was performed to determine the purity of the extracted CPS samples. The samples were stained with serially diluted LPS and BSA standards. Crude *E. coli* UB1005 culture supernatants were also stained to reflect the content in unpurified, non-enzyme-treated supernatants. Both CPS samples contained a distinct band with low molecular

TABLE 1 The effect of LPS on the minimum inhibitory concentration of Polymyxin B for E. coli strain CWG655

Strain	MIC value	LPS Concentration (in µg/mL)			
		20	10	5	0
CWG655	Polymyxin B (µg/mL)	5.0	2.5	2.5	0.08

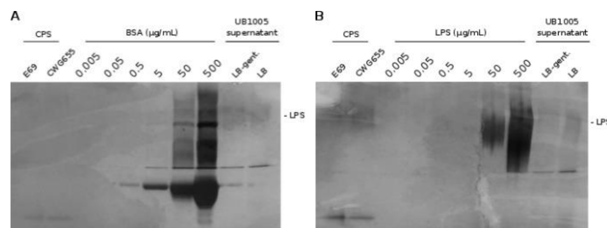

FIG. 2 Detection of LPS and protein contamination by silver staining. (A) Silver stain of CPS samples with BSA standard. Lane 1, *E. coli* E69 cell-free CPS sample; lane 2, *E. coli* CWG655 cell-free CPS sample; lane 3-8, serially diluted BSA standard; lane 9, supernatant of *E. coli* UB1005 culture in LB-gentamicin; lane 10, supernatant of *E. coli* UB1005 culture in LB. 40µL of samples were loaded on lane 1-8. 30µL of samples were loaded on lane 9-10. **(B)** Silver stain of CPS samples with BSA standard. Lane 1, *E. coli* E69 cell-free CPS sample; lane 2, *E. coli* CWG655 cell-free CPS sample; lane 3-8, serially diluted LPS standard; lane 9, supernatant of *E. coli* UB1005 culture in LB-gentamicin; lane 10, supernatant of *E. coli* UB1005 culture in LB. 40µL of samples were loaded on lane 1-8. 15µL of samples were loaded on lane 9-10.

weights despite previous enzymatic treatments and dialysis to digest and remove proteins and nucleotides (Fig. 2A). No similar bands were seen in the LPS standard, BSA standard, or the unpurified *E. coli* UB1005 supernatants. Furthermore, despite extensive ultracentrifugation, silver staining revealed a band in the CPS samples that was resolved at similar molecular weight as the LPS standard (Fig. 2B). Similar bands were also seen in the *E. coli* UB1005 supernatants. The bands also demonstrated a smeared pattern characteristic of LPS. Based on band intensities of our samples and the LPS standard, both CPS samples contained more than 5 µg/mL of LPS contamination before any dilutions.

Soluble LPS is more potent than CPS in conferring resistance to polymyxin B. To determine if soluble CPS can potentially sequester polymyxin B and neutralize its effect on inhibiting cell growth, we performed a broth MIC assay. Here, CPS isolated from both strains was used and compared alongside LPS in MIC assay. We showed that spiking CPS isolated from both E69 and CWG655 to the polymyxin B treated cells led to a 4-fold increase in the MIC compared to the media control from 0.063 µg/mL to 0.25 µg/mL (Table 2). However, the effect of 7.5 µg/mL CPS was less than 5 µg/mL LPS, showing that LPS was more potent in neutralizing polymyxin B activity. Furthermore, there was no difference in the MIC of polymyxin B across all conditions between strains E69 and CPS mutant CWG655 (Table 2). This indicates that addition of soluble CPS may have no discernible effect on the activity of PmB as measured by MIC.

TABLE 2 The effect of CPS and LPS on the minimum inhibitory concentrations of polymyxin B on E. coli strains E69 and CWG655

Strain	MIC value	Assay Condition			
		CPS		LPS	Media
		E69 (7.5µg/mL)	CWG655*	(5µg/mL)	Control
CWG655	Polymyxin B (µg/mL)	0.25	0.25	1.0	0.063
E69	Polymyxin B (µg/mL)	0.25	0.25	1.0	0.063

DISCUSSION

Bacterial capsule is an extracellular layer of polysaccharide that provides resistance to various environmental stressors such as antibiotics. In this study, we looked at the role of soluble capsular polysaccharides in providing enhanced resistance against the cationic antibiotic polymyxin B. Because PmB is also known to bind to LPS, we first performed an assay to test the effects of LPS on inhibiting PmB activity (27). We confirmed that LPS was sufficient to cause a noticeable increase in the MIC of polymyxin B at 5 µg/mL for *E. coli* CWG655 cells, necessitating the depletion of LPS from our CPS samples via ultracentrifugation (Table 2). The PmB-neutralizing effect of LPS was not tested with *E. coli* E69 cells as the wild-type strain has not arrived from the Whitfield lab at the time.

In order to perform an MIC assay to determine the efficacy of *E. coli* K30 cell-free capsular polysaccharide as a PmB-neutralizing agent, we first quantified the CPS using the uronic acid-carbazole assay. Samples were assayed only once to conserve reagents and CPS. Although the standard curve generated contained data points that deviated from a linear trend, a line of best fit, based on previous work performed, was nonetheless used to estimate the uronic acid concentrations within our samples (23). The variances in the data can be a result of contaminations by dust or oxidants in the reagents or the test tubes, as evidenced by the presence of green-blue coloration in our samples upon the addition of carbazole reagents. The absorbance attributed to oxidant contaminations is equivalent at 530 nm and 920 nm (23). Therefore, to correct the errors introduced by oxidant contaminations in future experiments, we suggest subtracting the spectrophotometric reading at 920 nm from the 530 nm reading (23). The CPS quantification shows that while *E. coli* E69 produces more cell-free capsular polysaccharide than the supposedly CPS-deficient mutant *E. coli* CWG655, the mutant still produces a significant amount of CPS. This assay measures the concentration of uronic acid, a monomer building block of *E. coli* K30 CPS (4). However, glucose can also be detected with this carbazole reaction, albeit at a much lower sensivity; specifically, 60 µg of glucose can yield signal equivalent to 8 µg of uronic acid (17). As such, we postulate that this discrepancy results from the contamination of samples by other *E. coli* surface polysaccharide that contains uronic acid or glucose. Previous study shows that *E. coli* E69, and by extension, *E. coli* CWG655, does not produce colonic acid (21). Furthermore, *E. coli* CWG655 has been shown to produce no immunoreactive K30 group 1 CPS (19). In addition, *E. coli* O9a antigen polysaccharide does not contain any uronic acid (28). However, it has come to our attention that the LPS outer core oligosaccharides are rich in glucose (29). Specifically, *E. coli* E69 and its derivatives has LPS core type R1, which contains five molecules of glucose in outer core (30). As such, we postulate that the centrifugation step, which involves a 16 hr spin at 47,000 x g followed by a 5 hr spin at 105,000 x g, was inadequate to fully pellet the LPS in the samples. Part of the loose LPS pellets was dislodged when the supernatants were collected. Thus, we suggest that the uronic acid signal detected in CWG655 supernatant extract is a result of cross-reactivity of carbazole with glucose from the LPS outer core oligosaccharides.

To sterilize the CPS, we selected filters with 0.45 µm pore size; however, significant reduction of CPS concentrations was observed, possibly due to CPS binding to cellulose acetate. In particular, virtually no uronic acid signal was detected in the filtered CWG655 extracted sample by the uronic acid-carbazole reaction; however, whether this is a signal artifact or a genuine result of depletion of CPS to an undetectable concentration by filtration can not be determined at this stage. In the subsequent assays, we treated the filtered CWG655 CPS sample as CPS-negative and diluted the sample using the same dilution factors for *E. coli* E69 CPS sample. Essentially, the filtered CWG655 CPS sample was used as a background for LPS contamination in the subsequent assays.

In previous studies, 10 µg/mL of *K. pneumoniae* K2 CPS has been shown to greatly increase the IC_{50} of PmB on *E. coli* C600 (14). Due to limited sample quantities, we supplemented the assay media with 7.5 µg/mL of E69 CPS, diluted from the resuspended, lyophilized samples. Our data suggest that the extracted CPS confers only limited polymyxin B resistance as measured by minimal inhibitory concentration assays. However, based on the same assays, more effective neutralization of polymyxin B is achieved with a lower concentration of lipopolysaccharide. In addition, no PmB MIC difference was observed between the wells supplemented with E69 CPS samples and the ones with CWG655 CPS samples, which, as previously mentioned, served as a LPS background. Finally, silver staining has shown that, while our CPS samples are relatively free of protein or nucleic acid contamination, the samples contain a non-negligible amount of LPS contamination due to inadequate ultracentrifugation process (Fig. 2). As a result, in the MIC assays, the final concentration of LPS contamination in the CPS is estimated to be within 0.5-5 µg/mL based on band intensities on silver stain gels and the dilution factors. As such, it is difficult to tease apart CPS- and LPS-mediated polymyxin B neutralization by examining the MIC assay readout. The observed shift in PmB MIC can be attributed entirely to the presence of LPS or to a combined effect of LPS and CPS.

In summary, we have shown that soluble LPS may play a more dominant role as a PmB-neutralizing agent than cell-free CPS does; given that the observed PmB-neutralizing effect of CPS is not as pronounced as that of

LPS at a lower, albeit still physiologically relevant, concentration, and that the limited PmB-neutralizing effect of CPS we observed may be, at least in part, mediated by the LPS contaminants in the CPS samples, contrary to previous findings (13, 14). To account for this discrepancy, we examined the structures of the CPS used in previous studies and in our experiments. Prior studies focused primarily on *Klebsiella pneumoniae* K2 CPS, whereas our experiments examined *E. coli* K30 CPS, whose composition and primary structure is highly similar to those of *K. pneumoniae* CPS, in that *E. coli* K30 CPS is composed of repeated tetrasaccharide containing mannose, galactose, and β-D-glucuronic acid, whereas the *K pneumoniae* K2 CPS consists of repeated tetrasaccharide containing mannose, glucose, and glucuronic acid (17, 31). Furthermore, it was postulated that the exact structures and subunits of the polysaccharide are of lesser concern, since the interactions between the cationic antimicrobial peptides, such as polymyxin B and human neutrophil α-defensin 1 (HNP-1), and the capsular polysaccharide are mediated by nonspecific electrostatic interactions (14). However, a more recent study demonstrates that both the LPS O-antigen and the charge-neutral group 4 *E. coli* capsular polysaccharide, which consists of the same sugar repeats as the O-antigen, are capable of binding and neutralizing human α-defensin 5 and HNP-1 (32). This, combined with the previous observation that LPS O-antigen deficient mutants *K. pneumoniae* are more susceptible to antimicrobial peptides even though the LPS lipid A moiety is modified, lends support to the theory that bacterial resistance to cationic peptides is not simply mediated by any single component of LPS or CPS, but by a combination of them, and likely via different mechanisms in different organisms despite the structural homologies between some of the components (13, 32). For example, both lipid A and CPS can potentially bind and neutralize cationic peptides; whereas the O-antigen as well as the CPS can also serve as shield against antimicrobial peptides (13, 14, 27, 32). Therefore, it is of interest to tease apart the exact role of each component in the antimicrobial peptide resistant gram-negative bacteria.

FUTURE DIRECTIONS

Future experiments should be conducted to further confirm our observations. To provide statistical significance, additional replicate experiment should be performed. Additionally, although we showed that LPS is more potent than CPS in providing increased resistance to polymyxin B, we were unable to accurately determine the isolated effect of CPS on neutralizing polymyxin B because of LPS contamination within our purified CPS samples. To resolve this, we suggest optimizing the ultracentrifugation process and accurately quantify any possible LPS with a standardized LPS detection kit. Alternatively, purified CPS from a commercial source, if available, may be used. Moreover, it may prove to be reasonable to pre-incubate the purified CPS with polymyxin B before adding cells to favour polymyxin B binding to free CPS rather than the cellular surface. This may result in an increase in the observed efficacy of CPS mediated neutralization of polymyxin B.

On the other hand, to ascertain the biological roles of *E. coli* CPS in the mediation of antimicrobial peptide resistance, future experiments should examine whether *E. coli* behaves similarly to *K. pneumoniae*, increasing shedding of soluble CPS when exposed to sublethal dose of polymyxin B or other cationic peptides. In the similar vein, because polymyxin B is an antibiotic which binds to the lipopolysaccharide layer of the outer membrane of gram-negative bacteria and disrupts membrane integrity which causes shedding of the bacterial exopolysaccharide, it would be interesting to test other membrane disrupting antibiotics and evaluate if soluble CPS, LPS, or the many various exopolysaccharides can act as a competitive inhibitor to antibiotic mediated destabilization of the outer membrane (14).

Finally, despite prior knowledge of the possible mechanisms of actions of LPS and CPS in the neutralization of polymyxin B, the exact contributions of each component of these polysaccharides are not well understood. Future experiments may aim to resolve the interactions between LPS or CPS with cationic peptides at a finer level, examining the roles and mechanisms of lipid A, O-antigen, and CPS in the binding or shielding of polymyxin B. For example, the PmB-neutralizing effect of CPS can be tested in a different *E. coli* background that lacks the O-antigen, has greatly reduced lipid A moiety charge due to covalent modifications, or both. Alternatively, one can test the role of exogenous O-antigen-truncated or full LPS in the neutralization of polymyxin B in capsule-deficient *E. coli* strains.

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