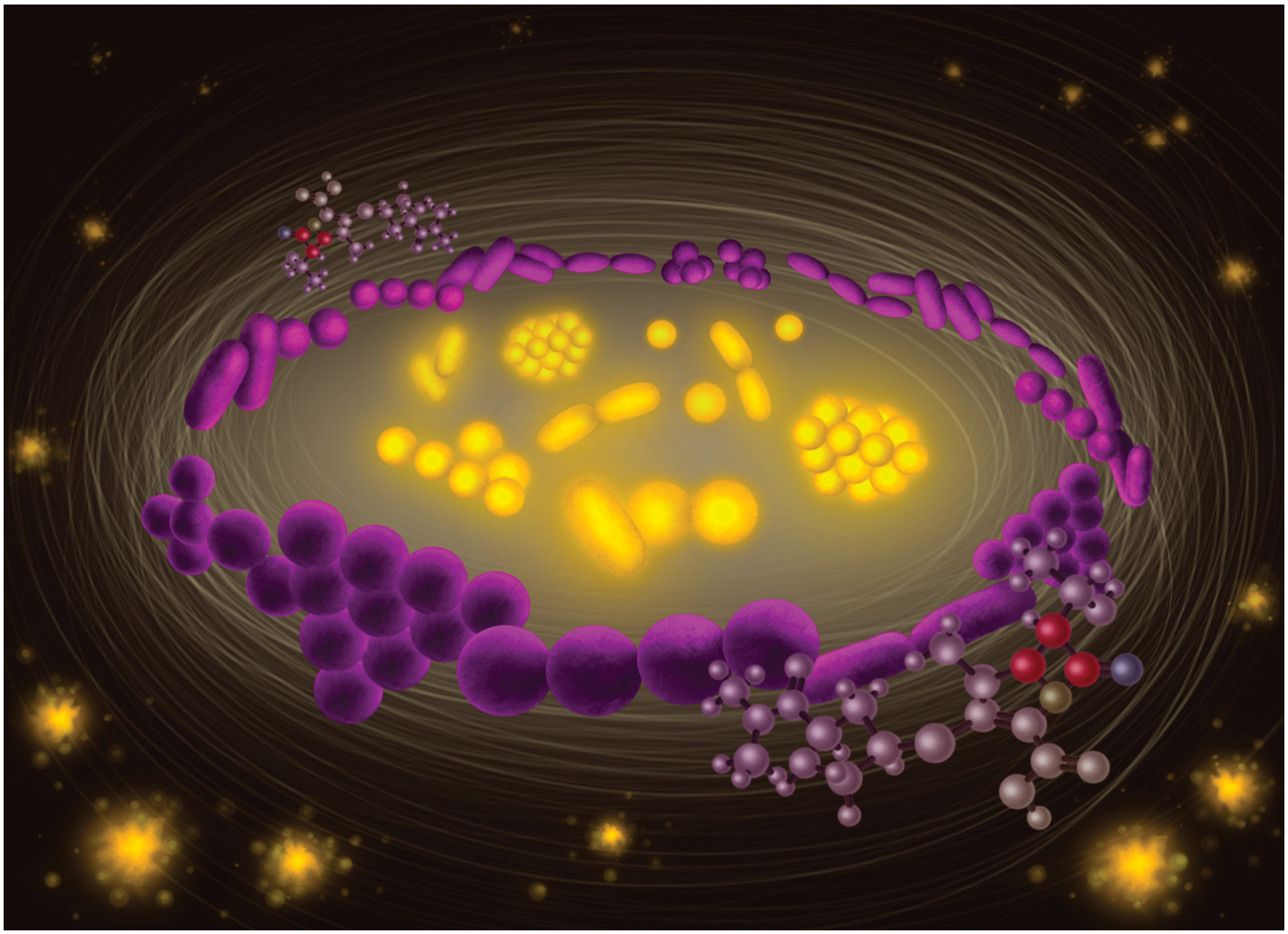

Showcasing research from Professor Lohans' laboratory, Department of Biomedical and Molecular Sciences, Queen's University, Canada.

Contributions of β-lactamase substrate specificity and outer membrane permeability to the antibiotic sheltering of β-lactam-susceptible bacteria

The production of β-lactamase enzymes by resistant bacteria can deplete β-lactam concentrations, helping β-lactam-susceptible bacteria to survive antibiotic exposure. We report the application of a luminescent reporter strain to quantify this phenomenon, using this method to evaluate the contributions of enzymatic and cellular factors to antibiotic sheltering. In addition to β-lactamase substrate specificity, the stability and permeability of the cell wall play major roles in determining the level of sheltering that occurs. Our findings inform on how the presence of resistant bacteria can impact antibiotic efficacy in a polymicrobial environment.

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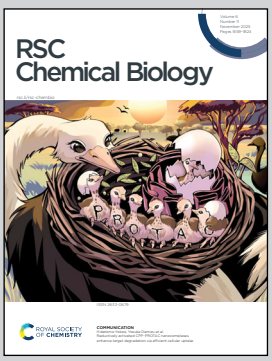

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Contributions of β-lactamase substrate specificity and outer membrane permeability to the antibiotic sheltering of β-lactam-susceptible bacteria

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The use of β-lactam antibiotics is threatened by antibiotic resistant bacteria that produce β-lactamases. These enzymes not only protect the bacteria that produce them but also shelter other bacteria in the same environment that would otherwise be susceptible. While this phenomenon is of clinical significance, many of the factors that contribute to β-lactamase-mediated antibiotic sheltering have not been well-studied. We report the development of a luminescence assay to directly monitor the survival of β-lactam-susceptible bacteria in the presence of β-lactamase-producing bacteria and β-lactam antibiotics. This method provides a rapid and scalable means of quantifying antibiotic sheltering in mixed microbial populations. We applied this assay to investigate the contributions of several factors to sheltering, including the class of β-lactam, the substrate specificity of the β-lactamase, and the cell wall permeability of the β-lactamase-producing bacterium. Our results show that the extent of sheltering that occurs not only depends on the particular combination of β-lactam and β-lactamase, but is also greatly impacted by the ability of a β-lactamase to access its β-lactam substrates.

Introduction

Microbes, including the trillions that live in the human body, exist in complex environments which can contain a multitude of different strains and species.[1] While many members of the human microbiota are beneficial to their host, the growth of pathogenic microbes in the body can lead to infections. Furthermore, some infections can result from the presence of multiple different pathogenic microorganisms.[2] These polymicrobial infections are frequently associated with a number of different health conditions, including cystic fibrosis, chronic obstructive pulmonary disease, wound infections, and dental cavities.[2–7] The co-occurrence of multiple pathogens has been shown to impact microbial processes such as colonization and virulence, and can complicate antimicrobial therapy.[5,8] Studies have shown that the ability of an antibiotic to target a bacterial pathogen can be influenced greatly by the other bacteria that are present in the same environment.[9–11]

Bacterial infections are commonly treated with β-lactams, a group which represents more than 50% of the antibiotics prescribed worldwide.[12–14] The members of this group, including the penicillins, cephalosporins, and carbapenems, contain a characteristic four-membered β-lactam ring which confers them with bactericidal activity. β-lactams target bacterial penicillin-binding proteins (PBPs), disrupting cell wall synthesis by inhibiting the last steps of peptidoglycan formation, ultimately leading to cell lysis.[14] When used to target Gram-negative bacteria, β-lactams must cross the outer membrane (OM) to reach the periplasmic space where PBPs are located (Fig. 1A).

Several different resistance mechanisms can protect bacteria against β-lactams, and the production of β-lactamases is especially prevalent among Gram-negative pathogens.[15–17] β-lactamase enzymes hydrolytically inactivate β-lactam antibiotics, preventing them from targeting PBPs and disrupting peptidoglycan synthesis. More than 8000 β-lactamase variants have been identified to date,[18] and the members of this group vary greatly in terms of which β-lactams they can degrade. Penicillinases like TEM-1 are principally active against penicillins and some cephalosporins, while extended-spectrum β-lactamases (ESBLs) like CTX-M-15 and SHV-2 can also degrade later generations of cephalosporins.[19,20] Carbapenemases such as NDM-1, IMP-1, and KPC-2 are a significant concern as they hydrolyse carbapenems, a group of β-lactams often reserved as treatments of last resort for the management of antibiotic-resistant infections.[21,22]

While the substrate specificity of a β-lactamase is determined by the structure of its active site and catalytic mechanism,

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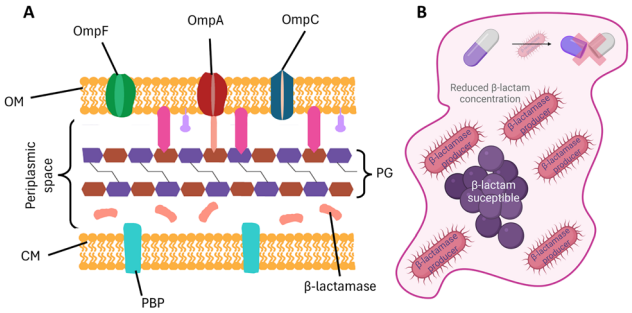

Fig. 1 (A) Overview of the major components in the Gram-negative cell envelope. β-lactams target penicillin-binding proteins located in the periplasm. Outer membrane porin proteins such as OmpF and OmpC allow β-lactams to enter the periplasm, where they can be degraded by β-lactamase enzymes. CM: cytoplasmic membrane; OM: outer membrane; PBP: penicillin-binding protein; PG: peptidoglycan. (B) Schematic representation of antibiotic sheltering. β-lactamase-producing bacteria (red) can protect bacteria that are susceptible to β-lactams (purple) by decreasing the local concentration of β-lactam antibiotic present in the extracellular environment. Created in BioRender (2025). https://BioRender.com/el5zavf.

β-lactamase activity is also impacted by the surface layers of the bacterium that produces it. As β-lactamases are primarily located in the periplasmic space in Gram-negative bacteria (Fig. 1A), β-lactam degradation in the periplasm first requires the antibiotic to cross the OM. The entry of many β-lactams is dependent on porins, protein channels in the OM that facilitate the entry of small molecules into the periplasm.[23,24] Hence, changes to porins and other outer membrane proteins may impact the rate of β-lactam degradation.

The production of β-lactamases by pathogenic bacteria can contribute to treatment failure when β-lactam antibiotics are used to treat polymicrobial infections.[12,25] This likely arises, at least in part, from the protection that β-lactamase-producing bacteria provide to β-lactam-susceptible bacterial populations (Fig. 1B).[26–34] By depleting the amount of extracellular β-lactam that is present, resistant bacteria can help susceptible bacteria survive antibiotic exposure.[35,36] This phenomenon has been the subject of many recent studies, and has often been referred to as antibiotic sheltering, bacterial cheating, collective resistance, or group beneficial traits.[26,35,37–41]

Although antibiotic sheltering is relevant in the context of the treatment of bacterial infections, most antibiotic susceptibility testing (in both academic and clinical contexts) uses bacterial monocultures. Indeed, the study of sheltering can be challenging, requiring the quantification of susceptible bacteria within a more complex population. To date, many studies in this area have employed plate counting,[27,42,43] dual flask experiments,[26,41] and growth cultures paired with mathematical models.[9,38,40] However, these methods can be labour-intensive, and differentiating between resistant and susceptible cells is sometimes challenging, particularly for phenotypically similar strains. Furthermore, some methods use a relatively complex experimental set up with continuous supplementation of components.[39,44] These drawbacks can complicate the use of such methods for the investigation of the factors that contribute to antibiotic sheltering.

We report the application of a luminescent *Escherichia coli* reporter strain to the characterization of the antibiotic sheltering provided by β-lactamase-producing bacteria. Sheltering assays using this reporter are fast and versatile, allowing for factors such as β-lactamase identity and cell wall permeability to be quantitatively investigated. We observed that the extent of sheltering is largely determined by the substrate specificity of the β-lactamase being produced, and is closely related to the kinetic rate of β-lactam degradation. OM permeability of the β-lactamase-producing strain also had a major impact on sheltering, and *E. coli* mutants lacking certain porins offered far less protection. Our methods and results will support future studies in this area which may inform on how the treatment of bacterial infections is impacted by the resistance mechanisms associated with other microbes present in the body.

Results and discussion

To investigate the factors that underlie the antibiotic sheltering provided by β-lactamase-producing bacteria, we prepared a luminescent β-lactam-susceptible *E. coli* BW25113 reporter strain transformed with the pJ23100LUX plasmid (Fig. S1). This strain constitutively expresses the *lux* operon, providing a rapid means for evaluating its growth and survival. Initial validation experiments showed that luminescence intensity is related to the number of bacterial cells present, and luminescence and OD600 measurements followed similar trends during the growth of *E. coli* BW25113 pJ23100LUX (Fig. S2). Addition of the β-lactam antibiotics meropenem or imipenem to the culture led to a decrease in luminescence over time reflecting the killing of the reporter strain (Fig. S3).

We next tested whether *E. coli* cells producing the β-lactamase KPC-2 can rescue the luminescent *E. coli* reporter strain from β-lactam antibiotics. A sample containing the reporter strain, a KPC-2-producing strain, and the carbapenem imipenem emitted a strong luminescent signal, while a sample in which the KPC-2-producing strain was substituted with a non-β-lactamase-producing *E. coli* strain emitted very low levels of luminescence (Fig. 2A). These measurements are consistent with the KPC-2-producing strain sheltering the reporter strain by degrading imipenem.

Testing multiple β-lactam concentrations in parallel allowed for dose–response curves to be obtained, as shown in sheltering experiments with the carbapenem meropenem and an *E. coli* strain that produces the β-lactamase NDM-1 (Fig. 2B). These dose–response data allowed for the determination of the meropenem concentration at which the growth of the β-lactam-susceptible strain [measured in relative luminescence units (RLU)] is inhibited by half (*i.e.*, an EC_{50} value). Different cell densities of the NDM-1 producing strain were tested for their ability to shelter the reporter strain against a range of meropenem concentrations. As expected, the EC_{50} values showed that higher numbers of NDM-1-producing bacteria led to increased levels of sheltering. β-lactamase expression levels were also shown to impact the level of sheltering, with *E. coli* cells

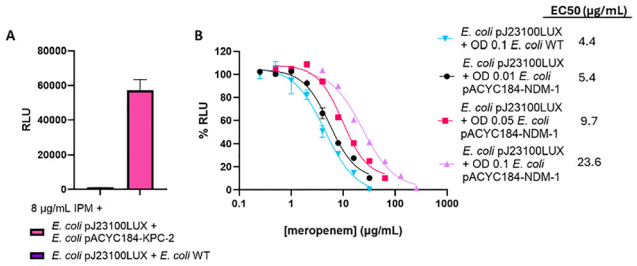

Fig. 2 Validation of the luminescence-based sheltering assay. (A) Luminescence measurements obtained from the sheltering assay when the reporter strain was treated with 8 μg mL^{-1} of imipenem in the presence of KPC-2-producing bacteria and non-β-lactamase-producing bacteria. n = 4. (B) Dose–response curves showing the impact of different meropenem concentrations on the luminescence of the reporter strain in the presence of different cell densities of NDM-1-producing *E. coli*. 95% confidence intervals are reported in Table S1. n = 3. RLU: relative luminescence units; IPM: imipenem; WT: wild-type. Error bars represent standard deviations.

transformed with the high copy pHSG298-NDM-1 plasmid sheltering the reporter strain from meropenem at a level approximately three times greater than that seen for *E. coli* transformed with the lower copy pACYC184-NDM-1 (Fig. S4).

Following these initial experiments, we carried out assays evaluating the level of sheltering provided by a panel of *E. coli* strains producing different clinically relevant β-lactamases against selected β-lactam antibiotics. Of the β-lactamases tested, NDM-1, KPC-2, IMP-1, and OXA-48 are carbapenemases,[21,45,46] while TEM-116 is a penicillinase and CTX-M-15 is an ESBL.[47] EC_{50} values were determined for selected combinations of β-lactamases and β-lactams, with a focus on sheltering involving the carbapenems meropenem and imipenem. While β-lactamases with carbapenemase activity can degrade carbapenems, these antibiotics are not efficiently degraded by penicillinases and ESBLs.[48,49]

Most of the carbapenemase-producing *E. coli* strains dramatically improved the survival of the reporter strain in the presence of meropenem, with the extent of sheltering depending on the identity of the carbapenemase (Fig. 3, Table S1). While *E. coli* producing NDM-1 and KPC-2 were observed to increase the EC_{50} values by five- and eight-fold, respectively, when compared to non-β-lactamase-producing *E. coli* (Fig. 3A and B), IMP-1-producing *E. coli* increased the EC_{50} value by more than 10-fold (Fig. 3C). However, the EC_{50} values for the unsheltered controls exhibited some variability, complicating the comparison of enzymes. Although OXA-48-producing *E. coli* provided almost no sheltering, with just a 1.5-fold increase in EC_{50} (Fig. 3D), this is consistent with the relatively low catalytic activity of OXA-48 against carbapenems.[50,51] As expected, *E. coli* producing the penicillinase TEM-116 did not increase the EC_{50} when compared to the unsheltered control (Fig. 3E).

We related the sheltering assay results to β-lactamase activity by carrying out UV-vis spectrophotometric assays measuring the degradation of meropenem by the β-lactamase-producing bacteria tested (Fig. 3F). Of the strains tested, the NDM-1-producing *E. coli* degraded meropenem most quickly and yielded the highest EC_{50} value in the sheltering assays. The *E. coli* strains

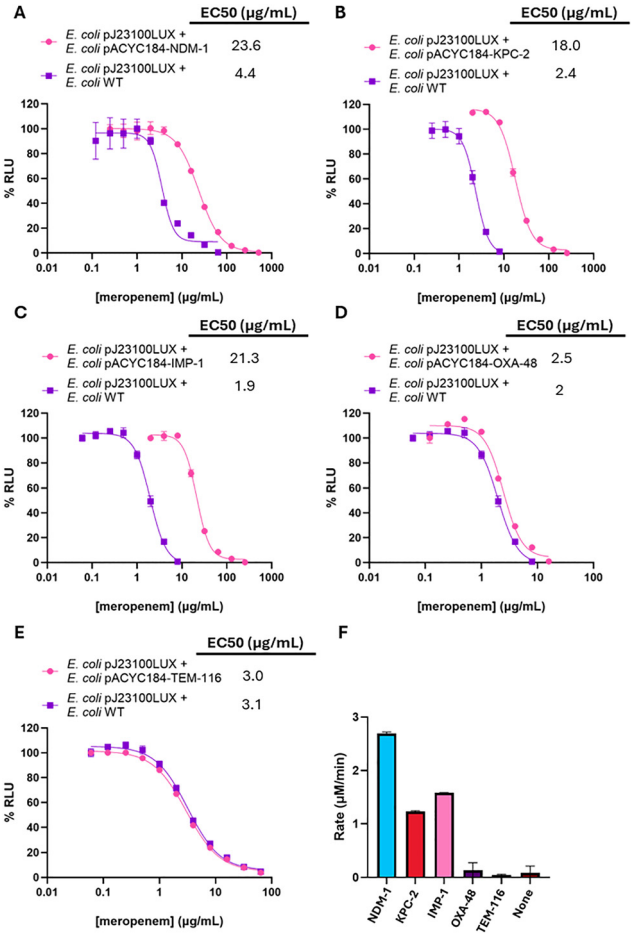

Fig. 3 Sheltering of the reporter strain by β-lactamase-producing *E. coli* against the carbapenem meropenem. Dose–response curves used to determine EC_{50} values for the luminescent reporter strain in the presence of meropenem and *E. coli* strains producing (A) NDM-1, (B) KPC-2, (C) IMP-1, (D) OXA-48, and (E) TEM-116. (F) Initial velocities for meropenem (167 μM) hydrolysis by β-lactamase-producing cells as determined by UV-vis spectrophotometry. 95% confidence intervals for dose–response curves are reported in Table S1. %RLU: percent normalized relative luminescence units. n = 4. Error bars represent standard deviations.

producing KPC-2 and IMP-1 degraded meropenem more slowly than NDM-1, which may explain the smaller EC_{50} values obtained for the strains producing these enzymes. The very low levels of meropenem hydrolysis catalysed by *E. coli* producing OXA-48 and TEM-116 was consistent with the poor levels of sheltering observed.

Carbapenemases vary in terms of how efficiently they degrade different carbapenems. Exploring this specificity, we carried out sheltering assays with the carbapenem imipenem in place of meropenem. Similar to what was seen with meropenem, *E. coli* strains producing the carbapenemases NDM-1 and KPC-2 sheltered the susceptible reporter strain from imipenem, increasing the EC_{50} values by more than six-fold compared to a non-β-lactamase producing control (Fig. 4A, B and Table S1). As with meropenem, some variability was observed in the EC_{50} values for the unsheltered control experiments. Although the OXA-48-producing strain offered less protection (Fig. 4C and Table S1),

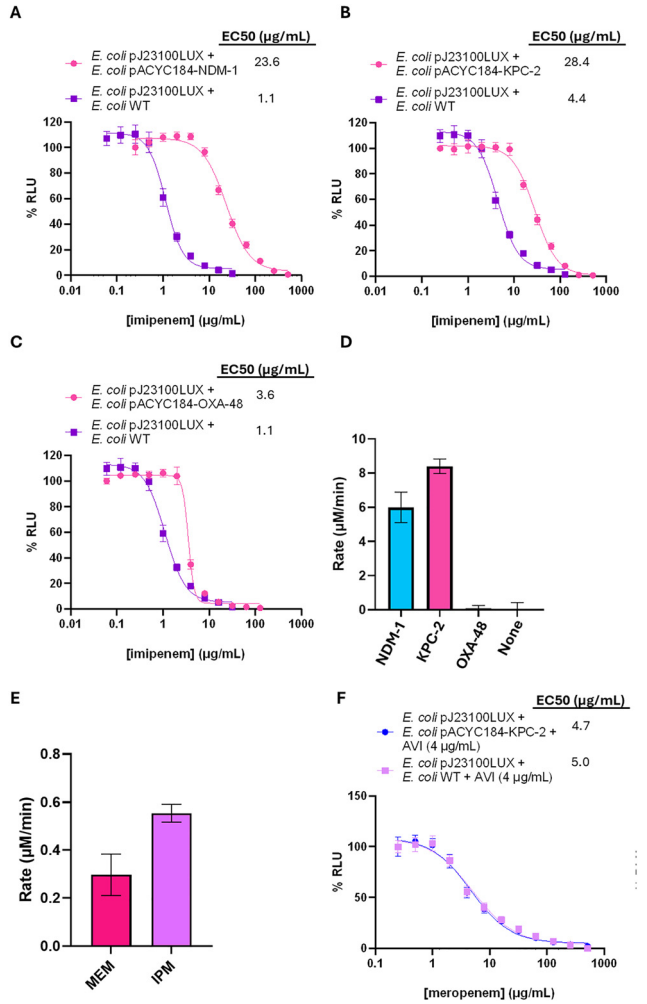

Fig. 4 Sheltering of the reporter strain by carbapenemase-producing *E. coli* against the carbapenem imipenem. Dose–response curves used to determine the EC_{50} values for the luminescent reporter strain in the presence of imipenem and *E. coli* strains producing (A). NDM-1, (B). KPC-2, and (C). OXA-48. (D) Initial velocities for imipenem (214 μM) hydrolysis by β-lactamase-producing cells as determined by UV-vis spectrophotometry. (E) Initial velocities for meropenem and imipenem (both 80 μM) hydrolysis using a higher density of OXA-48-producing cells, as determined by UV-vis spectrophotometry. (F) Dose–response curves showing that the EC_{50} value for KPC-2-producing *E. coli* treated with meropenem and the β-lactamase inhibitor avibactam is similar to that obtained for non-β-lactamase-producing *E. coli*. 95% confidence intervals for dose–response curves are reported in Table S1. IPM: imipenem; AVI: avibactam; % RLU: percent normalized relative luminescence units. $n = 4$. Error bars represent standard deviations.

the fold increase in EC_{50} for imipenem was greater than what was seen for meropenem and OXA-48. The general trends observed in these imipenem sheltering assays align with the initial velocities of imipenem hydrolysis for NDM-1-, KPC-2- and OXA-48-producing *E. coli* strains measured by UV-vis spectrophotometry (Fig. 4D). Due to slow substrate turnover, a higher cell density was needed when testing the kinetics of the OXA-48-producing strain; consistent with previous reports describing the substrate specificity of OXA-48,[52,53] these cells degraded imipenem more quickly than meropenem (Fig. 4E).

β-lactam antibiotics are often prescribed in combination with β-lactamase inhibitors as a countermeasure against β-lactamase-producing bacteria. There are several clinically approved inhibitors which target serine β-lactamases (SBLs) (*e.g.*, KPC-2), preventing these enzymes from degrading β-lactam antibiotics.[13] To test whether the presence of a β-lactamase inhibitor impacts the level of sheltering that occurs, we carried out a sheltering assay with KPC-2-producing *E. coli*, the β-lactamase inhibitor avibactam,[54] and meropenem (Fig. 4F). The addition of 4 μg mL^{-1} avibactam prevented measurable sheltering from occurring, lowering the EC_{50} for a sample containing KPC-2-producing *E. coli* to that observed for a non-β-lactamase-producing control (Fig. 4F and Table S1).

Although our studies were primarily focused on sheltering involving carbapenems and carbapenemase-producing bacteria, we tested whether the assay could be applied to investigate antibiotic sheltering involving other types of β-lactams. Bacteria that produce ESBLs are a major clinical concern because of the ability of these enzymes to hydrolyse extended-spectrum cephalosporins such as ceftriaxone.[55,56] We tested the sheltering offered by *E. coli* producing the ESBL CTX-M-15 against cefazolin (first generation cephalosporin) and ceftriaxone (third generation cephalosporin) (Fig. 5A, B and Table S1). Comparable levels of sheltering were observed with these two cephalosporins, with an approximately two-fold increase in EC_{50} values occurring in both instances, when compared to a non-β-lactamase-producing control. The similar levels of sheltering provided by CTX-M-15 align with previous kinetic studies

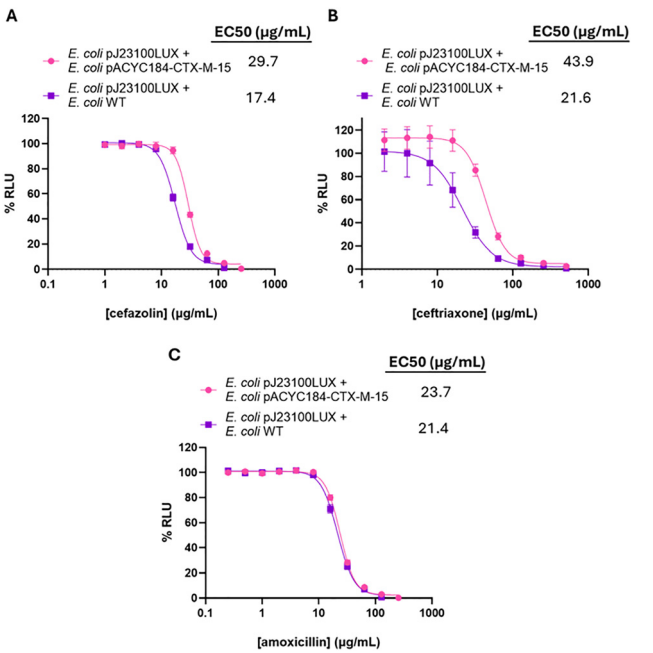

Fig. 5 Sheltering experiments with cephalosporins and penicillins. Dose–response curves showing the EC_{50} values for the reporter strain in the presence of CTX-M-15-producing *E. coli* cells and (A) cefazolin, (B) ceftriaxone, and (C) amoxicillin. 95% confidence intervals are reported in Table S1. %RLU: normalized percent relative luminescence units. $n = 4$. Error bars represent standard deviations.

reporting similar k_{cat}/K_M values for purified CTX-M-15 with cefazolin and ceftriaxone.[57] However, the CTX-M-15-producing *E. coli* provided little-to-no sheltering against amoxicillin (Fig. 5C and Table S1), consistent with the relatively low catalytic efficiency for CTX-M-15 against this penicillin.[58,59]

Our results have demonstrated the major role of β-lactamase substrate specificity in antibiotic sheltering. However, the rate of β-lactam hydrolysis catalysed by β-lactamases located in the periplasm of a Gram-negative bacterium will also depend on how easily a β-lactam antibiotic can enter the periplasm (Fig. 1A).[60] The passage of β-lactams across the Gram-negative OM often depends on porin proteins which form transmembrane channels.[61,62] In *E. coli*, OmpC and OmpF are non-specific porins used by a variety of antibiotics to enter the cell.[63] Gram-negative bacteria often become resistant to antibiotics through mutations to porin-encoding genes, reducing the entry of antibiotics such as β-lactams into the periplasm.[64] To investigate whether changes to antibiotic entry impact β-lactamase-mediated antibiotic sheltering, we tested NDM-1-producing *E. coli* mutants in which the *ompF*, *ompC*, and *ompA* genes were disrupted.

In these sheltering assays, the NDM-1-producing Δ*ompC* and Δ*ompA E. coli* strains were observed to have greater EC_{50} values when compared to wild-type (WT) NDM-1-producing *E. coli* (Fig. 6A). This suggests that these mutant strains in fact provide greater levels of sheltering compared to the wild-type strain. Consistent with this observation, both Δ*ompC* and Δ*ompA* strains degraded meropenem more quickly than the WT strain in a kinetic β-lactamase assay (Fig. 6B). Although the Δ*ompA* and Δ*ompC* strains exhibited similar rates of meropenem degradation, the Δ*ompC* strain provided more sheltering than the WT strain, while the differences in EC_{50} values for the WT and Δ*ompA* strains may not be statistically significant (Table S1).

The porin OmpC has been shown to play an important role in the entry of carbapenems,[65] and strains overexpressing OmpC exhibit lower minimum inhibitory concentrations (MICs) for carbapenems.[66] While this would suggest that the Δ*ompC* strain should degrade meropenem more slowly, disruption of the *ompC* gene has been demonstrated to destabilize the OM.[61] This likely leads to increased β-lactam entry and consequently greater antibiotic degradation and sheltering. This proposal was supported by an *N*-phenyl-1-naphthylamine (NPN) OM permeability assay (Fig. 6C), where the greater fluorescence intensity observed for the Δ*ompC* strain is consistent with OM destabilization.

OmpA plays a major role in the stability of the *E. coli* cell wall by non-covalently anchoring the OM to peptidoglycan.[67] Loss of OmpA compromises the OM, rendering bacteria more susceptible to stresses including antibiotics.[61,67] Similar to what was seen with the Δ*ompC* strain, the loss of OmpA may allow more β-lactams into the periplasm, or it could potentially increase the release of β-lactamases into the extracellular environment. Our NPN assay showed increased fluorescence for the Δ*ompA* strain as compared to the WT strain (Fig. 6C). However, the lower fluorescence intensity of the Δ*ompA* strain compared to the Δ*ompC* strain may indicate less OM destabilization occurs, following the same trend as seen in the sheltering assays.

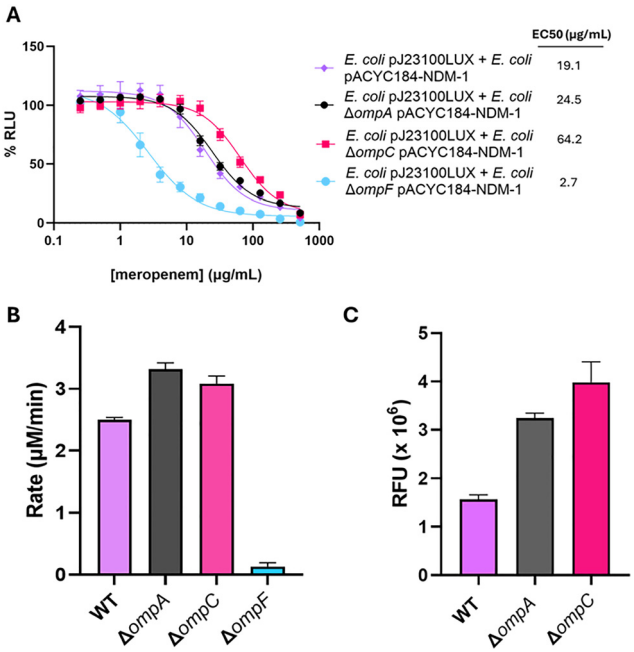

Fig. 6 Meropenem sheltering and hydrolysis assays for NDM-1-producing *E. coli* BW25113 mutants lacking porins. (A) Dose–response curves showing the EC_{50} values determined for the reporter strain in the presence of meropenem and NDM-1-producing *E. coli* WT, Δ*ompA*, Δ*ompC*, and Δ*ompF*. 95% confidence intervals are reported in Table S1. (B) Initial velocities of meropenem (80 μM) hydrolysis by *E. coli* strains (WT and variants) transformed with pACYC184-NDM-1, as determined by UV-vis spectrophotometry. (C) Outer membrane permeability assay measuring *N*-phenyl-1-naphthylamine (NPN) uptake by *E. coli* BW25113 WT, Δ*ompA*, and Δ*ompC*. % RLU: percent normalized relative luminescence units. RFU: relative fluorescence units. n = 3. Error bars represent standard deviations.

Based on the relationship between OM destabilization and antibiotic sheltering, lysis of the β-lactamase-producing bacteria was expected to provide high levels of antibiotic sheltering. Indeed, a lysate of NDM-1-producing bacteria yielded a high EC_{50} value when measuring the survival of the reporter strain in the presence of meropenem (Fig. S5). Although the extent of sheltering afforded by these lysed cells did not appear to be as great as what was seen for the Δ*ompC* strain, the results are not directly comparable; the β-lactamase-producing bacteria normally grow throughout the sheltering assay, increasing the amount of β-lactamase present, whereas the amount of enzyme in the lysed sample is fixed.

In contrast to the other two mutants, the Δ*ompF* strain provided far lower levels of sheltering with an EC_{50} that was six-fold lower than that observed for wild-type NDM-1-producing *E. coli* (Fig. 6A and Table S1). OmpF is used by carbapenems and other β-lactams to enter the periplasm,[68,69] and disrupting the *ompF* gene increases the MICs of carbapenems.[70,71] In our UV-vis β-lactamase assays, the NDM-1-producing Δ*ompF E. coli* strain degraded meropenem approximately 20-times slower than the WT *E. coli* strain (Fig. 6B). With less meropenem reaching the periplasm of the NDM-1-producing

bacteria, less antibiotic degradation occurs, exposing the susceptible reporter strain to higher antibiotic concentrations. Notably, the loss of OmpF is frequently observed in clinical isolates as an antibiotic resistance mechanism.[68]

Our findings show that antibiotic sheltering depends not only on the specific β-lactam-β-lactamase combination under investigation, but also on cellular factors that influence the ability of β-lactamases to interact with their targets. Beyond differences in porin expression, factors such as efflux pumps and lipopolysaccharide structure will also impact the accumulation of antibiotics in the periplasm. As such, the extent of sheltering provided by clinically relevant β-lactamase-producing bacteria likely differs from what we observed testing lab strains.

We next extended our sheltering assays to the study of three carbapenemase-producing clinical isolates (Fig. 7). A VIM-producing *Enterobacter cloacae* strain provided the highest level of sheltering with an EC_{50} value of 19 μg mL^{-1}, while a KPC-2-producing *Klebsiella oxytoca* strain and an NDM-producing *Klebsiella pneumoniae* strain yielded similar EC_{50} values of 10 μg mL^{-1} and 11 μg mL^{-1}, respectively (Fig. 7A and Table S1). In these experiments, the non-β-lactamase-producing *E. coli* ATCC25922 strain served as a negative control and offered no sheltering. The results obtained from these sheltering assays followed similar trends as seen from UV-vis kinetic analyses of meropenem hydrolysis, where the *E. cloacae* strain demonstrated the fastest rate of hydrolysis, followed by the *K. pneumoniae* and *K. oxytoca* strains (Fig. 7B). The results obtained for these clinical isolates are more moderate than what was seen for laboratory strains which produce similar β-lactamases, suggesting that differences in permeability, efflux, and β-lactamase expression levels are likely decreasing the level of sheltering that occurs.

Antibiotic sheltering is a phenomenon that should be considered when defining the treatment course for a bacterial infection, particularly if there is a polymicrobial etiology. The presence of multiple bacterial pathogens with different antibiotic resistance mechanisms can lead to treatment failure and severe complications.[12] Furthermore, resistance genes can be harboured by commensal microbes,[72] and the products of these microbes could influence antibiotic treatment. Consequently, it is critical to recognize the impact that bacterial production of antibiotic-degrading enzymes can have on the survival of antibiotic-susceptible bacteria in the same environment. We show that, under the assay conditions tested, β-lactamase-producing bacteria can allow β-lactam-susceptible bacteria to survive antibiotic concentrations far greater than what would normally kill them.

While our luminescence-based assay has proven to be an effective method for investigating antibiotic sheltering, there are certain limitations. During our studies, we observed that increases in cell density reduced the luminescence measurements, presumably because cells interfere with the ability of light to reach the detector. However, this limitation could be addressed by optimizing the incubation period to prevent overgrowth and ensure reliable luminescence measurements. Another limitation is that the *in vitro* conditions used do not reflect the environmental conditions at the site of an infection (*e.g.*, cell density, richness of growth medium). Nonetheless, our assay offers a fast and quantitative approach for investigating the factors that contribute to antibiotic sheltering. This strategy could be extended to study interactions involving more complex bacterial populations, sheltering in the context of biofilms, or the sheltering provided by other antibiotic-degrading enzymes.[35]

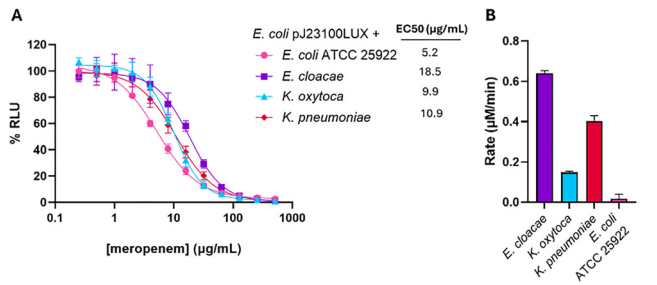

Fig. 7 Meropenem sheltering and hydrolysis assays for β-lactamase-producing clinical isolates. (A) Dose–response curves showing the EC_{50} values determined for the *E. coli* ATCC25922 non-β-lactamase-producing control, a VIM-producing *E. cloacae* strain, a KPC-producing *K. oxytoca* strain, and an NDM-producing *K. pneumoniae* strain in the presence of meropenem. These assays measured the sheltering of the luminescent *E. coli* BW25113 pJ23100LUX reporter strain. 95% confidence intervals are reported in Table S1. (B) Initial velocities for meropenem (80 μM) hydrolysis by the clinical strains and *E. coli* ATCC25922 as determined by UV-vis spectrophotometry. %RLU: percent normalized relative luminescence units. n = 3. Error bars represent standard deviations.

Conclusions

β-lactamase-producing bacteria can greatly increase the survival of susceptible bacteria following exposure to β-lactam antibiotics. Consistent with previous reports,[9,26,43] we observed that sheltering depends greatly on the particular combination of β-lactam and β-lactamase. Our work also establishes new insights into the major role that OM permeability and stability have in the process of antibiotic sheltering.

The combination of multiple resistance mechanisms (*e.g.*, β-lactamase production and loss of OmpF) can drastically impact the extent of sheltering that occurs. This is significant, as clinical isolates often harbour multiple resistance mechanisms that, when combined, greatly decreases their susceptibility to antibiotics.[73–75] Our findings emphasize the importance of evaluating the resistance mechanisms of all of the microbes present in the environment of an infection, particularly in the context of polymicrobial infections. By establishing a reproducible, quantitative assay for studying sheltering, our methodology enables future investigations into more complex and clinically relevant microbial communities with diverse resistance mechanisms.

Our study focused on β-lactamase activity in the periplasm, but these enzymes can also be released into the extracellular environment as the result of cell lysis or through packaging in outer membrane vesicles.[76] Future studies will further

investigate how the localization of β-lactamases influences the extent of antibiotic sheltering. Additionally, further research will examine the impact of environmental factors and other cell wall-targeting antibiotics on sheltering.

Materials and methods

Reagents and materials

Sterile 2TY media (16 g L^{-1} tryptone, 10 g L^{-1} yeast extract, and 5 g L^{-1} sodium chloride) was used to culture bacteria, with tryptone and yeast extract purchased from BioShop and sodium chloride from Fisher Scientific. Meropenem trihydrate, imipenem, cefazolin, amoxicillin, kanamycin monosulfate salt and ceftriaxone disodium salt hemi(heptahydrate) were purchased from Glentham Life Sciences. Chloramphenicol and NPN were purchased from Fisher Scientific. BugBuster Protein Extraction Reagent was purchased from Millipore Sigma.

Bacterial strains and plasmids

The plasmid pJ23100LUX was prepared by cloning the *luxCDABE* operon (amplified from pAKgfplux2; Addgene #14083) between the NcoI and SacI restriction sites of pIDMv5K (provided by Sebastian Cocioba), placing the operon under the control of the strong constitutive J23100 promoter (Fig. S1). *E. coli* BW25113 transformed with pJ23100LUX was used as a luminescent β-lactam-susceptible reporter strain.

The gene and promoter for CTX-M-15 were amplified from the genomic DNA of a *Klebsiella pneumoniae* strain by polymerase chain reaction (PCR) and cloned into pACYC184 (National BioResource Project, NBRP) similar to our previous report.[77] The gene and promoter for NDM-1 were amplified from pACYC184-NDM-1 by PCR and cloned into the EcoRI site of pHSG298 (NBRP) by in-cell ligation.[77] *E. coli* BW25113 and single-gene knockout mutants from the Keio collection (Δ*ompA*, Δ*ompC*, and Δ*ompF*) were obtained from the NBRP.[78,79] β-lactamase-producing lab strains were prepared by transforming *E. coli* BW25113 and the single gene knockout mutants with pACYC184 or pHSG298 plasmids encoding β-lactamases NDM-1, KPC-2, TEM-116, IMP-1, CTX-M-15, and OXA-48.[77] Clinical isolates were provided by the clinical microbiology lab at Kingston General Hospital (KGH).

Growth and antibiotic killing curves

The β-lactam-susceptible *E. coli* BW25113 pJ23100LUX reporter strain was grown overnight in 2TY media containing 50 μg mL^{-1} kanamycin at 37 °C and 200 rpm. A subculture was prepared by inoculating (1%) fresh 2TY (supplemented with 50 μg mL^{-1} kanamycin) with the overnight culture, which was then incubated at 37 °C and 200 rpm. The growth curve was determined by collecting luminescence and optical density (OD) measurements every hour using a Synergy LX multimode plate reader (Agilent BioTek). Clear non-treated Falcon 96-well microplates (Corning) were used for OD measurements. White LUMITRAC 200 96-well plate (Greiner Bio-One) were used for luminescence measurements. Killing curves were generated following the same protocol, except 2 μg mL^{-1} meropenem or imipenem was added to the subculture at time 0. Quadruplicates of each sample were measured.

Enzyme kinetics

β-lactamase activity was measured by UV-vis spectrophotometry. *E. coli* expressing NDM-1, KPC-2, IMP-1, OXA-48, TEM-116, or CTX-M-15 was cultured on 2TY agar plates supplemented with 25 μg mL^{-1} chloramphenicol. Suspensions of these *E. coli* were prepared in Dulbecco's phosphate-buffered saline (DPBS) to an OD600 of 0.15. Stock solutions of meropenem and imipenem were prepared in distilled water. In a 96-well UV-Star microplate (Greiner Bio-one), 100 μL of antibiotic solution and 100 μL of cell suspension were mixed and the change in absorbance at 297 nm over time was measured using a Synergy LX multimode plate reader (Agilent BioTek). Initial velocities were obtained by plotting antibiotic concentrations against time, and a linear regression fit was calculated using GraphPad Prism 10.2.3. Extinction coefficients of 11 500 M^{-1} cm^{-1} and 10 940 M^{-1} cm^{-1} were used for imipenem and meropenem, respectively.[80]

NPN outer membrane permeability assays

A 5 mM solution of NPN was prepared in acetone, then diluted with sterile water to 0.5 mM. *E. coli* BW25113 wild-type (WT), Δ*ompA*, and Δ*ompC* were cultured on 2TY agar overnight at 37 °C. Colonies were suspended in DPBS to a OD600 of 0.3, and 200 μL of each suspension was added to the wells of a black FLUOTRAC 200 96-well plate (Greiner Bio-One). To each well, 4 μL of 0.5 mM NPN was added. Fluorescence measurements (excitation 350 nm, emission 420 nm) were taken after 5 min using a Spectramax ID3 multimode plate reader (Molecular Devices).

Sheltering assays

Untransformed *E. coli* BW25113, the *E. coli* BW25113 pJ23100LUX luminescent reporter strain, and the β-lactamase-producing *E. coli* BW25113 strains were cultured on 2TY agar (supplemented with kanamycin or chloramphenicol as needed) overnight at 37 °C. Unless otherwise stated, colonies were suspended in liquid 2TY to an OD600 of 0.1. Serial dilutions of the antibiotics tested were prepared in 2TY media. In a white LUMITRAC 200 96-well plate (Greiner Bio-One), 100 μL of antibiotic solution was mixed with a 100 μL suspension of a β-lactamase-producing strain and a 100 μL suspension of the reporter strain. The plate was incubated at 37 °C and luminescence was measured using a Synergy LX multimode plate reader after one hour. Control samples without β-lactamase-producing bacteria consisted of 100 μL antibiotic, 100 μL of the reporter strain suspension, and a 100 μL suspension of untransformed *E. coli* BW25113. Luminescence values were blanked and normalized within each data set and plotted against antibiotic concentration. For sheltering assays with lysed cells, a cell density of NDM-1-producing *E. coli* corresponding to an OD600 of 0.1 was suspended in 30% BugBuster for 10 min, then diluted two-fold with 2TY. Using diluted lysate in place of

intact β-lactamase-producing cells, assays were otherwise carried out as described above. Dose–response curves were obtained using a non-linear fit analysis in GraphPad Prism 10.2.3 for the reporter strain with a β-lactamase producing strain (sheltered), and for the reporter strain with an untransformed strain (not sheltered).

Author contributions

M. M.-O.: conceptualization, formal analysis, investigation, methodology, validation, visualization, writing – original draft, writing – review and editing. M. A. J.: investigation, writing – review and editing. J. L. L.: investigation, writing – review and editing. C. T. L.: conceptualization, funding acquisition, project administration, resources, supervision, writing – review & editing.

Conflicts of interest

There are no conflicts of interest to declare.

Data availability

The data supporting this article have been included as part of the SI. See DOI: https://doi.org/10.1039/d5cb00092k

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