The carbapenem inoculum effect provides insight into the molecular mechanisms underlying carbapenem resistance in *Enterobacterales*

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Abstract

Carbapenem-resistant *Enterobacterales* (CRE) are important pathogens that can develop resistance via multiple molecular mechanisms, including enzymatic hydrolysis or reduced antibiotic influx. The identification of these mechanisms is crucial for effective pathogen surveillance, infection control, and patient care. However, many clinical laboratories do not test for the molecular basis of resistance. In this study, we investigated whether we could gain insight into resistance mechanisms by using the inoculum effect (IE), a phenomenon where the inoculum size used in antimicrobial susceptibility testing (AST) affects the minimum inhibitory concentration (MIC) measured. We demonstrated that seven different carbapenemases impart a meropenem IE when expressed in *Escherichia coli*. Next, we measured the meropenem MIC as a function of inoculum size for 110 clinical CRE isolates. We found that the carbapenem IE was strictly dependent on the resistance mechanism: carbapenemase-producing CRE (CP-CRE) exhibited a strong IE, whereas porin-deficient CRE (PD-CRE) displayed none. Strains with both carbapenemases and porin deficiency had higher MICs at low inoculum and also an IE; we termed these "hyper-CRE". Concerningly, 50% and 24% of CP-CRE isolates changed susceptibility classification to meropenem and ertapenem, respectively, across the allowable inoculum range in clinical guidelines, with 42% testing meropenem susceptible at some point in this range. The meropenem IE and the ratio of ertapenem to meropenem MIC at standard inoculum reliably distinguished CP-CRE and hyper-CRE from PD-CRE. Understanding how molecular mechanisms of resistance affect AST could improve diagnosis and guide therapies for CRE infections.

Significance Statement

Infections caused by carbapenem-resistant *Enterobacterales* (CRE) pose a significant threat to public health worldwide. Carbapenem resistance can occur through several molecular mechanisms, including enzymatic hydrolysis by carbapenemases and reduced influx via porin mutations. Knowing the mechanisms of resistance informs therapies and infection control measures to prevent further spread of these deadly pathogens. In a large collection of CRE isolates, we found that only carbapenemase-producing CRE exhibit an inoculum effect, in which their measured resistance varies markedly with cell density, which risks misdiagnosis. Measuring this inoculum effect, or integrating other data from routine antimicrobial susceptibility testing, enhances the detection of carbapenem resistance and thus paves the way for more effective strategies to combat this growing public health crisis.

Main Text

Introduction

Carbapenems are a crucial class of β-lactam antibiotics with a broad spectrum of activity (1) due to their ability to withstand hydrolysis by many β-lactamases and their inhibition of cell wall synthesis across many bacterial species (2). The emergence of carbapenem-resistant *Enterobacterales* (CRE) poses a significant public health risk, with the US Centers for Disease Control and Prevention (CDC) designating them as an “urgent threat” due to their extensive drug resistance, high mortality rates, and treatment challenges (3, 4). The rapid global emergence of CRE has created an urgent clinical and epidemiological need to better understand, detect, and limit the spread of these isolates (5).

CRE employ two major molecular mechanisms of carbapenem resistance: 1) expression of carbapenemases, which efficiently hydrolyze carbapenems or 2) disruption of porins, which reduces influx of carbapenems into the periplasm where they act (6). This reduced periplasmic influx can potentiate β-lactamases with weak intrinsic carbapenemase activity such as AmpC, CTX-M, SHV, TEM, or OXA-10, and together cause resistance (1, 7). Carbapenemase-producing CRE (CP-CRE) are of particular epidemiological concern due to their propensity to cause outbreaks (4, 8) and their ability to transfer carbapenemases to other bacteria (9–11). Porin deficient CRE (PD-CRE) often exhibit impaired fitness (12) compared to CP-CRE because porins play important roles in nutrient import (13), osmotic homeostasis (12, 14), and membrane lipid asymmetry (15). Determining resistance mechanisms could lead to better infection control measures, such as patient isolation to prevent further CP-CRE spread in hospitals (4, 11, 16). Some laboratories have started to test for resistance mechanisms in CRE (17–19), but this can be burdensome due to additional time, equipment, and expertise required. Capitalizing on

existing antimicrobial susceptibility testing (AST) workflows to predict mechanisms of resistance would represent a clinical advance in the diagnosis of these critically important pathogens.

Standardized AST methods and interpretive criteria are critically important to determine the susceptibility of pathogens. Organizations like the Clinical and Laboratory Standards Institute (CLSI) classify pathogen susceptibility based on minimum inhibitory concentration (MIC) breakpoints (20–22) that are established based on the available microbiological, pharmacokinetic-pharmacodynamic, and clinical data. These breakpoints are proactively evaluated and revised as new data emerge (23). Despite recently updated breakpoints (24), some CRE can still be misclassified as susceptible to carbapenems in clinical laboratories.

The inoculum effect (IE) is a phenomenon in which the measured MIC varies depending on the precise bacterial inoculum used in AST assays, which can lead to susceptibility misclassification and treatment failures (25, 26). Multiple factors may contribute to the IE, including metabolic state of the bacteria (27), growth productivity (28), antibiotic-target ratio (29), heteroresistance frequency (30), target proteolysis (31), and production of enzymes that inactivate antibiotics (32–34). The IE is frequently observed for some antibiotic classes including β-lactams (35) and more recently antimicrobial peptides (36), but not commonly for others such as aminoglycosides. While importance of the IE in clinical infections remains uncertain (37, 38), it has important implications for diagnostic accuracy. To minimize the impact of the IE, CLSI recommends an inoculum of 5×10^5 colony forming units (CFU)/mL with an acceptable range of 2×10^5 to 8×10^5 CFU/mL for broth microdilution, which is considered the gold standard AST method (39). However, even within this range, carbapenem susceptibility classification of *Enterobacterales* isolates may change due to the IE, underscoring the need for continued efforts to optimize AST methods (40).

Here, we sought to systematically investigate how different genotypes conferring carbapenem resistance influence the IE and how understanding these relationships might be leveraged to improve clinical AST. We found that expression of distinct carbapenemases was

sufficient to cause a marked meropenem IE in a laboratory strain of *E. coli*. Further, in a collection of 110 clinical CRE isolates across 12 species of *Enterobacterales* (9, 41), we observed that the carbapenem IE was a function of mechanism of resistance. Specifically, all CP-CRE exhibited a strong carbapenem IE, whereas all PD-CRE exhibited constant carbapenem MICs across a wide range of inocula. Isolates with both a carbapenemase and porin deficiency generally exhibited an IE and higher MICs than strains with either mechanism alone. The carbapenem MICs of CP-CRE varied considerably, with a large proportion changing AST classification across the CLSI-acceptable inoculum range. Collectively, our results demonstrated that the IE distinguished CRE by resistance mechanism, better than either meropenem or ertapenem MIC alone. Leveraging these findings, we identified two phenotypic assays that predict carbapenem resistance mechanism better than current MIC cutoffs.

Results

Carbapenemases belonging to all four Ambler classes impart an inoculum effect.

Carbapenemases are commonly divided into four classes based on protein sequence and enzymatic features (42, 43). To investigate if each carbapenemase class is sufficient to confer an IE, we systematically cloned seven different carbapenemase genes, each with its native promoter, into a low-copy pBAD33 plasmid (44): $bla_{\text{KPC-3}}$ and $bla_{\text{SME-2}}$ from Ambler class A; $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, and $bla_{\text{VIM-27}}$ from class B; $bla_{\text{CMY-10}}$ (45) from class C; and $bla_{\text{OXA-48}}$ from class D (*SI Appendix*, Table S1). We transformed each carbapenemase-encoding plasmid into *E. coli* K-12 MG1655, a carbapenem-susceptible laboratory strain, then conducted meropenem broth microdilution MIC assays across a broad range of inocula, comprising two-fold dilutions from 1.3×10^7 to 1.6×10^3 CFU/mL. As inoculum increased from low (light grey) to high (black), all transformants showed a significant increase in MIC, while the MIC of the empty vector control did not increase significantly (Fig. 1*A*). Most transformants had stable meropenem MICs from

~10^3 to ~10^4 CFU/mL, and all exhibited a strong linear increase in MIC across the ~10^5 to ~10^7 CFU/mL inoculum range (Fig. 1*B*). In this strain background, NDM-1 (orange), SME-2 (pink), and IMP-4 (brown) imparted the highest levels of resistance, followed by VIM-27 (yellow). KPC-3 (teal) bestowed high-level resistance at high inocula, but within the CLSI inoculum range (dashed lines in Fig. 1*B*) MICs remained within the intermediate classification (>1 μg/mL and <4 μg/mL). Two carbapenemases generated unique characteristics: first, NDM-1 conferred unusually high resistance at low inocula compared to all other carbapenemases. Second, SME-2 imparted MICs that started increasing from ~10^3 CFU/mL. OXA-48 (violet) and CMY-10 (green) conferred the lowest meropenem MICs, which remained below 1 μg/mL until the highest inoculum tested and well within the susceptible classification in the CLSI range.

Inoculum effect of clinical CRE isolates depends on resistance mechanism. After establishing that all carbapenemases imparted an IE in *E. coli* K-12, we investigated the IE in clinical isolates harboring carbapenemases, porin disruptions, or both. We analyzed a group of 110 clinical isolates comprising six genera and 12 species (Table S2), which we assigned to three groups based on their genotypes: CP-CRE, PD-CRE, and CP-CRE with porin deficiency (Fig. 2 and *SI Appendix*, Fig. S1, Table S2 and Table S3). We observed that all 36 CP-CRE harboring carbapenemases from one of six major families (KPC, NDM, IMP, VIM, OXA-48, and SME) displayed an IE similar to the *E. coli* K-12 carbapenemase transformants (Fig. 2*A* and *SI Appendix*, Fig. S1*A*). Some trends seen in *E. coli* K-12 transformants were also observed in these isolates, such as NDM (orange) imparting the highest MICs at low inocula and the onset of IE at low inocula in isolates encoding SME (Fig. 2*A* and *SI Appendix*, Fig. S1*A*). In contrast, 47 PD-CRE exhibited a strikingly different pattern of resistance, with nearly constant MICs across the tested inoculum range (Fig. 2*B* and *SI Appendix*, Fig. S1*B*). Finally, the 27 isolates with both carbapenemase production and porin deficiencies exhibited an IE and higher-level resistance than isolates containing only one of the two mechanisms of resistance (Fig. 2*C* and

SI Appendix, Fig. S1*C*). Because of this higher-level resistance, we henceforth refer to isolates with both a carbapenemase and porin deficiency as “hyper-CRE”.

Carbapenemase production and porin deficiency together cause a hyper-CRE phenotype. To directly test the hypothesis that the combination of carbapenemase production and porin deficiency confers a hyper-CRE phenotype, we selected two clinical *K. pneumoniae* isolates (BIDMC46a and BIDMC91) with disruptions in both major porins OmpK36 and OmpK35, and transformed them with a pBAD33 plasmid encoding a KPC-3 (Fig. 3*A*). Both transformants exhibited a dramatic increase in MIC at standard inoculum (256-fold and 128-fold, respectively) compared to empty vector controls and also developed an IE (6-fold from standard to high inoculum). In a second experiment, we disrupted the major OmpK36 porin in a *K. pneumoniae* isolate expressing a KPC-2 (RB582) using a CRISPR-Cas9 base editor (46). Meropenem MICs at standard inoculum increased by 32-fold in the edited strain, with a small 2-fold IE (Fig. 3*B*). These experiments provide direct evidence that the combination of carbapenemase production and porin deficiency leads to a hyper-CRE phenotype.

A significant proportion of carbapenemase-producing isolates change classification within the CLSI inoculum range. Although clinical microbiological laboratories initially identified the isolates we studied as carbapenem-resistant, the IE caused the MIC of several isolates to vary greatly within the CLSI inoculum range (Fig. 2*A*, and *SI Appendix*, Fig. S1*A*). This variability prompted us to quantify which carbapenemase-producing isolates changed susceptibility classification within the acceptable CLSI inoculum range of 2×10^5 CFU/mL to 8×10^5 CFU/mL. 22 of 63 (35%) carbapenemase-producing isolates tested with meropenem and 7 of 41 (17%) tested with ertapenem changed AST classification (Fig. 4). When excluding hyper-CRE and considering only isolates with intact porins, 18 of 36 (50%) CP-CRE isolates changed AST classification for meropenem and 7 of 29 (24%) for ertapenem within the CLSI inoculum

range. Concerningly, three isolates changed from susceptible to resistant when tested with meropenem in the CLSI range, including one *E. coli* isolate (BIDMC43b) and two *S. marcescens* isolates (BWH56 and BWH57). Four isolates tested fully susceptible to meropenem throughout the CLSI inoculum range (red circle), including two *Citrobacter freundii* harboring KPC-3 (MGH281 and MGH283), and one *E. coli* (BAA2523) and one *K. pneumoniae* (BAA2524) each harboring an OXA-48. In all, 15 of 63 (24%) carbapenemase producers (CP-CRE or hyper-CRE) tested meropenem susceptible, and 2 of 41 (4.9%) tested ertapenem susceptible, at some point in the CLSI inoculum range; this represented 15 of 36 (42%) and 2 of 29 (6.9%) CP-CRE, respectively.

The inoculum effect accurately identifies carbapenemase production among CRE. The meropenem MIC at standard inoculum was ineffective at distinguishing carbapenemase-producing isolates (CP-CRE or hyper-CRE) from PD-CRE (Fig. 5*A*). However, the phenotypic differences between these groups across inocula led us to investigate a diagnostic approach based on the IE. We quantified the IE by dividing the MIC at high inoculum (1.3×10^7 CFU/mL) by the MIC at standard inoculum (4×10^5 CFU/mL) for each isolate (Fig. 5*B*). CRE exhibited distinct meropenem IEs depending on their mechanism of resistance, with carbapenemase-producing isolates (CP-CRE and hyper-CRE) exhibiting a much more prominent IE, with a median increase in MIC of 10-fold, compared to a 1.2-fold increase for PD-CRE (Fig. 5*B*). The meropenem IE distinguished carbapenemase-producing CRE from PD-CRE better than meropenem MIC at standard inoculum, as illustrated by ROC curves for each measurement (Fig. 5 *C* and *D*), with ROC area under the curve (AUC) of 0.99 for the meropenem IE versus 0.69 for the meropenem MIC alone. At one specific IE threshold of 2.53-fold, the sensitivity and specificity of distinguishing carbapenemase-encoding isolates from PD-CRE was 95.2% and 95.7%, respectively. The meropenem IE was greater in CP-CRE (median 16-fold MIC increase)

than hyper-CRE (median 5.3-fold), and it also distinguished these two groups (Fig. 5 *B* and *D*; ROC AUC 0.893).

Comparing ertapenem and meropenem MICs gives insights into resistance mechanisms. The meropenem IE effectively distinguished carbapenemase-producing isolates from PD-CRE, but it is not a standard workflow in clinical laboratories, which typically only perform AST at standard inocula. To overcome this limitation, since the periplasmic permeability of ertapenem is typically more impacted by porin mutations than that of meropenem (6, 7, 39, 47), we hypothesized that comparing ertapenem and meropenem MICs, even at a single inoculum, might also distinguish CRE by mechanism of resistance. We thus measured ertapenem MICs across a broad inoculum range for 85 of the 110 CRE isolates (Fig. 6). The ratio of ertapenem MIC to meropenem MIC better separated carbapenemase producing isolates (CP-CRE or hyper-CRE) from PD-CRE (Fig. 6*C*) than either the ertapenem or meropenem MIC alone (Fig. 6 *A* and *B*). At standard inoculum, meropenem MIC performed poorly in our collection of CRE, distinguishing carbapenemase producers (CP-CRE or hyper-CRE) from PD-CRE with a ROC AUC of 0.559, whereas ertapenem MIC performed slightly better with an AUC of 0.696 (Fig. 6*D*). By contrast, the ertapenem-to-meropenem MIC ratio yielded an AUC of 0.941, with a 90% sensitivity and 84% specificity at a ratio cutoff of 7.63 for detecting carbapenemase content. At low inoculum, ertapenem MIC performed comparably to this ratio at identifying carbapenemase producing isolates, while at high inoculum, meropenem MIC performed comparably. However, at the standard CLSI inoculum, neither performed as well as the MIC ratio.

Discussion

Our systematic investigations revealed that the *in vitro* carbapenem inoculum effect (IE) is not determined by the mechanism of action of the antibiotic, but rather by the mechanism of

resistance of the isolate. In the isogenic background of *E. coli* K-12, we showed that expression of any carbapenemase is sufficient to produce a carbapenem IE (Fig. 1). We confirmed this result in a collection of 110 clinical CRE isolates, where all CP-CRE exhibited a strong IE, whereas PD-CRE did not (Fig. 2, *SI Appendix,* Fig. S1, and Table S3). Isolates with both mechanisms of resistance (hyper-CRE) tended to have higher MICs and also exhibited an IE, although smaller in magnitude than CP-CRE (Fig. 2*C* and Fig. 5*B*).

Our findings have important mechanistic, clinical, and diagnostic implications. Mechanistically, our findings suggest that carbapenemases can act as a "common good" in bacterial populations, as has been proposed previously proposed for other secreted enzymes (32, 34), and that this is sufficient to explain the carbapenem IE. Furthermore, the biphasic dependence of CP-CRE MIC on inoculum (Fig. 1*B*, Fig. 2*A* and *SI Appendix*, Fig. S1*A*) implies that while carbapenemases confer resistance to the individual producing cell at low inoculum, beyond a certain cell density threshold, the collective production of carbapenemases becomes the dominant determinant of resistance. Notably, PD-CRE isolates expressing extended spectrum β-lactamases (ESBLs), which often contribute to carbapenem resistance, did not exhibit an appreciable carbapenem IE (Fig. 2*B* and *SI Appendix*, Fig. S1*B*), indicating that the slow rates of carbapenem hydrolysis by ESBL enzymes were insufficient to impact extracellular carbapenem concentrations enough to benefit neighboring bacteria even at high culture density. The relative rarity of carbapenemases may explain why the IE is seen less frequently for carbapenems than for other β-lactams (48), but this might change as these enzymes continue to spread globally. The mechanistic basis for carbapenemase release into the extracellular space where it can benefit neighboring cells, whether via secretion systems, outer membrane vesicles, cell lysis, or other means, warrants more investigation.

The clinical implications of the inoculum effect have been uncertain because the relevant bacterial cell densities during infection in vivo are generally below those used for AST (40, 49). For instance, in bacteremia, the infectious burden of ≤10 CFU/mL is several orders of

magnitude below that at which the inoculum begins to affect MIC in our assays (50, 51). Nevertheless, in *Staphylococcus aureus* bacteremia, a cefazolin IE has been associated with treatment failure and higher mortality (52, 53). Our findings suggest that at the low inoculum present in bacteremia, PD-CRE (Fig. 2*B* and *SI Appendix*, Fig. S1*B*) exhibit higher carbapenem MICs than CP-CRE (Fig. 2*A*, Fig. S1*A*), yet clinical outcomes are similar or worse for CP-CRE (54, 55). It is possible that the increased resistance of PD-CRE at physiologically relevant cell densities may be offset by fitness costs associated with reduced permeability. Further research is needed to fully understand the relationship between MIC, IE, resistance mechanisms, and clinical outcomes.

Diagnostically, accurate recognition of carbapenem resistance is crucial for infection control strategies and patient care. Our results confirmed prior reports that minor differences in inoculum can lead to categorical changes in susceptibility interpretation (40), particularly impacting the detection of CP-CRE, the isolates that carry the greatest implications for infection control. Even though the set of isolates we examined were selected because they tested as CRE, many changed classification within the CLSI-acceptable inoculum range and even appeared susceptible (Fig. 4), suggesting that clinical algorithms may miss some isolates encoding carbapenemases. Our observations in *E. coli* K-12 reveal that strains harboring OXA-48 or even KPC might only test resistant if they possess other factors, such as reduced antibiotic permeability (Fig. 1, Fig. 2, and *SI Appendix*, Fig. S1, Table S3). The clinical significance of isolates that express a carbapenemase but test susceptible in the standard inoculum range is uncertain and requires further study. Nonetheless, caution is generally advised when using carbapenems in infections with carbapenemase-producers, even if they test susceptible, due to the lack of evidence supporting clinical outcomes in these cases (20). Our findings in a set of isolates selected for meropenem nonsusceptibility suggest that other carbapenemase producers may have been missed by standard diagnostic workflows, which could contribute to poor patient outcomes and the global spread of carbapenem resistance.

The strong correlation between the carbapenem IE and genotype (Fig. 2) allows for improved predictions about resistance mechanisms from phenotypic testing. Our study revealed that the meropenem IE is a more sensitive and specific indicator of carbapenemase production in CRE than MIC at any single inoculum (Fig. 5). While carbapenemase detection is performed with molecular or enzymatic testing in some laboratories (17, 56, 57), this fully phenotypic trait could be used to prioritize deployment of these advanced tests. Our work could extend recent advances showing that molecular detection of carbapenemases can accelerate the administration of targeted therapies, such as β-lactamase inhibitor combinations (58), and decrease mortality (19). Moreover, this phenotypic approach could detect strains producing rare carbapenemases, such as SME or CMY-10, that are not routinely included in testing panels. This finding, in addition to its therapeutic value, could serve as a surveillance tool for the emergence and evolution of carbapenemases (59).

Although measuring the meropenem IE would require additional testing workflows, we also found that integrating ertapenem and meropenem MICs from routinely generated AST data can inform the likelihood of carbapenemase production (Fig. 6). The ratio of ertapenem to meropenem MIC predicts resistance mechanism better than either MIC alone, perhaps because the periplasmic permeability of ertapenem is more strongly affected by porin disruption than that of meropenem (6, 39, 47), while both are hydrolyzed at similar rates by carbapenemases (60, 61). Specifically, CRE with a high ratio of ertapenem to meropenem MIC (more resistant to ertapenem) are likely to be PD-CRE, whereas those with a low MIC ratio (similar resistance to both) are likely to be CP-CRE. As with the inoculum effect, hyper-CRE appear to fall in between, and tend to have higher MICs at any inoculum. Since the IE impacts both ertapenem and meropenem MICs similarly (Fig. 4), this ratio predicts carbapenemase production independent of inoculum, an advantage over either ertapenem or meropenem MICs alone (Fig. 6). We propose that this ratio might be better suited than either MIC alone to inform subsequent testing and prioritizing isolates for molecular carbapenemase detection. If its accuracy is validated on

independent isolate collections, this ratio could even inform empiric selection of additional agents while awaiting extended susceptibility testing and molecular diagnostics.

This study has several limitations and areas requiring further study. First, the clinical isolates analyzed were mostly chosen because they tested carbapenem resistant, limiting the ability to determine the frequency of CP-CRE isolates that may be missed by routine testing due to the IE. Further studies are needed to assess the performance of our proposed assays on isolates with MICs at or below the susceptible breakpoint. Second, automated AST instruments may not provide precise MIC measurements for ertapenem and meropenem outside a narrow range around the susceptibility breakpoints, requiring further investigation into the utility of lower-resolution MIC estimates in screening for CP-CRE. Third, while we found carbapenemases to be sufficient to confer a carbapenem IE, other factors such as heteroresistance, metabolic factors, quorum sensing, and target distribution may also contribute in some isolates or circumstances. However, carbapenemases predictably cause a robust IE without the involvement of other factors. In particular, our consistent observation of a linear increase in MIC with increasing inoculum (Fig. 2 and *SI Appendix*, Fig. S1) is not compatible with heteroresistance, the emergence of a rare discrete subpopulation from larger inocula. This would manifest as a stepwise, stable increase in MIC at an inoculum threshold, rather than a continuous increase in MIC with inoculum. Lastly, this study did not explore the basis for the IE for other antibiotics, nor how other processes like antibiotic efflux may contribute to carbapenem resistance. However, the consistent relationship between resistance mechanism and the IE for carbapenems, where hydrolytic enzymes and other resistance mechanisms are relatively few and well characterized, may offer a useful general model for the IE. Future studies could involve pairing these detailed phenotypic data and genotypic information (*SI Appendix*, Table S3) with precise enzymological parameters in order to enhance and experimentally test existing quantitative models (33, 49). This would enable a more comprehensive description of how

different mechanisms of antibiotic resistance impact the growth of bacterial populations when exposed to antibiotic selection.

Antibiotic resistance often reflects several molecular processes acting in concert. Carbapenemase content and porin status together explained the levels of carbapenem resistance in most isolates we tested. Some genera like *Klebsiella* appear to exhibit higher carbapenem MICs than others like *Citrobacter* even when they encode the same carbapenemase-containing plasmid (9). This may reflect baseline differences in permeability by phylogenetic grouping, which is consistent with general trends in the ertapenem-to-meropenem MIC ratio across species in our collection (*SI Appendix*, Table S3). However, some isolates were outliers, exhibiting unexpectedly high resistance or inoculum-dependent patterns that suggest uncharacterized elements contribute to their carbapenem resistance. In some cases, these unexpected behaviors were clues to simple errors in sample workup. For instance, in one isolate (MGH4), the deposited genome assembly did not reveal a carbapenemase (10) but it exhibited a strong meropenem MIC suggestive of a CP-CRE. We performed PCR and Sanger sequencing, revealing that MGH4 harbored a KPC, consistent with the IE. The plasmid was likely lost during strain passaging prior to whole genome sequencing. Detailed IE phenotype also allowed characterization of porin polymorphisms of uncertain significance, which are a frequent challenge for genotypic prediction of β-lactam resistance (62, 63). Even with the insight generated in this study, we observed that some of our CRE isolates had unexplained levels or patterns of carbapenem resistance across a broad range of inocula. Such outliers underscore remaining gaps in our knowledge and motivate further investigations to characterize unknown processes leading to high-level resistance, which might be encountered more frequently as CRE continue to spread globally.

Methods:

Bacterial isolates. The use and collection of bacterial strains from three major Boston-area hospitals (Beth Israel Deaconess Medical Center, BIDMC; Brigham and Women's Hospital, BWH; Massachusetts General Hospital, MGH) was approved by the Partners Health Care Institutional Review Board (IRB under protocol 2015P002215) and additional IRB approval was granted by the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects. Additional isolates were acquired through the University of California Irvine School of Medicine (UCI), the Wadsworth Center of the New York State Department of Health (NYSDOH), the FDA-CDC Antimicrobial Resistance Isolate Bank (CDC, Atlanta, GA), and the American Type Culture Collection (ATCC) (Manassas, VA).

Cloning of carbapenemases. $bla_{\text{KPC-3}}$, $bla_{\text{SME-2}}$, $bla_{\text{IMP-4}}$, $bla_{\text{NDM-1}}$, $bla_{\text{VIM-27}}$, and $bla_{\text{OXA-48}}$ along with their native promoters were cloned by Gibson assembly from isolates encoding these carbapenemases into a pBAD33 plasmid which encodes chloramphenicol resistance (*SI Appendix*, Table S1). $bla_{\text{CMY-10}}$ with its native promoter was synthesized in the same pBAD33 vector backbone by GeneWiz (Suzhou, China).

Broad-range broth microdilution. Our MIC assays were adapted from previously published broth microdilution methods(22, 64). See Supplemental Methods for details on setup of the broad inoculum range in this assay format.

Data analysis. MIC replicates were imported to GraphPad Prism v 9.5.1 (GraphPad Software Inc., San Diego, CA); MIC vs inoculum plots (Figs. 1, 2, and 3, and *SI Appendix*, Fig. S1) depict mean and standard errors of the mean. To analyze the meropenem IE, the ratio of mean MIC at 1.3×10^7 CFU/mL to 4×10^5 CFU/mL was calculated. The IE of the 47 porin deficient CRE strains were compared to the 63 carbapenemase-encoding CRE strains using a non-parametric test (Mann-Whitney for comparisons between two groups or Kruskal-Wallis for analysis of more

than two groups) in GraphPad Prism. Statistical analysis comparing PD-CRE isolates versus CP-CRE and hyper-CRE isolate at low, standard, and high inocula for ertapenem, meropenem, and ertapenem-to-meropenem MIC ratio was conducted using Mann-Whitney tests. All receiver operating characteristic (ROC) curves and analysis were constructed using GraphPad Prism.

Gene editing to disrupt OmpK36 in *Klebsiella pneumoniae* isolate RB582. A CRISPR-Cas9 cytidine base-editing system(46) was used to introduce an early termination codon in the intact *ompK36* of isolate RB582. See Supplemental Methods for further details.

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Figures

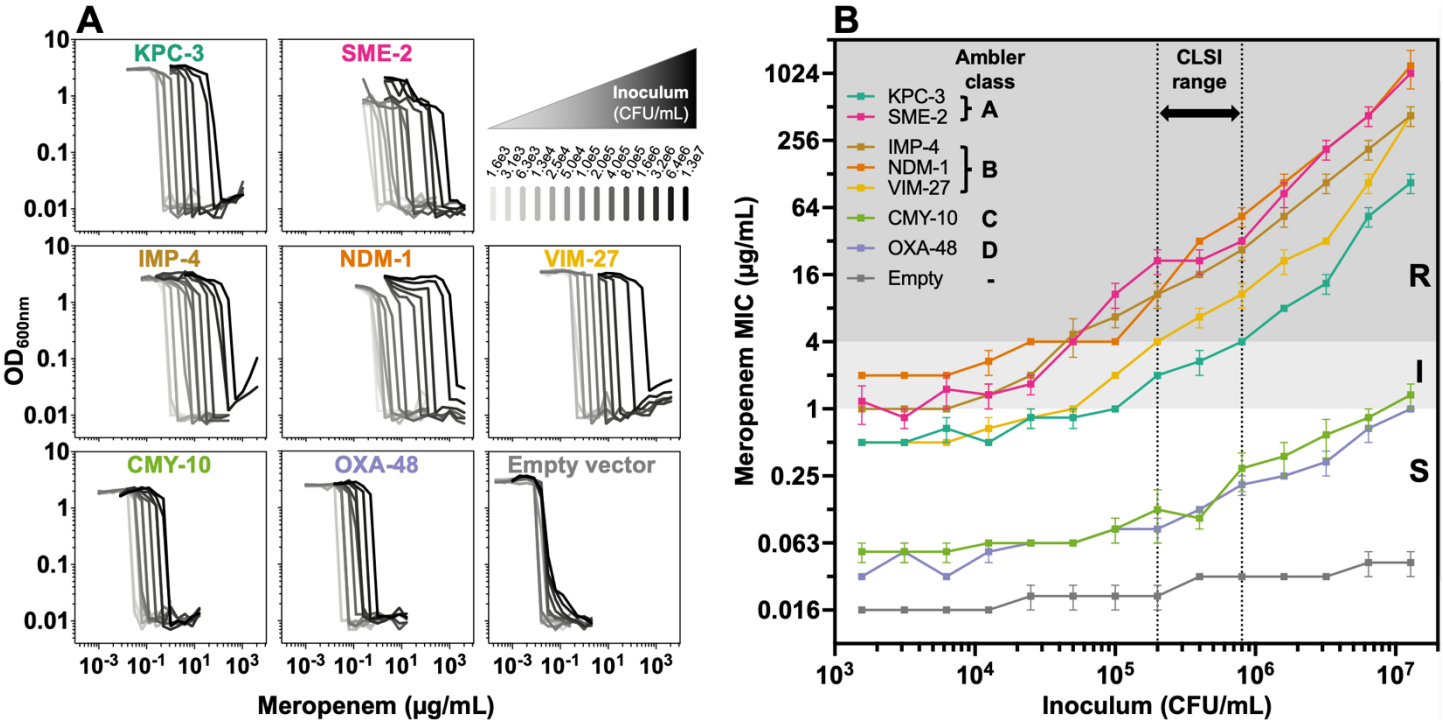

Fig. 1. All carbapenemases impart a meropenem inoculum effect when expressed in *E. coli K12*. (A) Plots of optical density (OD) at 600 nm (y-axis) after overnight incubation with varying meropenem concentrations (x-axis) across a broad range of starting inocula (grayscale per top right panel) for *E. coli* K-12 transformed with each indicated carbapenemase. **(B)** Meropenem minimum inhibitory concentration (MIC) as a function of bacterial inoculum, colored by transformed carbapenemase. Each MIC point is the mean of three replicates (error bars = standard error of the mean). Vertical dotted lines represent the CLSI-acceptable inoculum range (2 to 8 x 10^5 CFU/mL). The background is shaded by CLSI meropenem susceptibility breakpoints (S = susceptible, white; I = intermediate, light gray; R = resistant, gray).

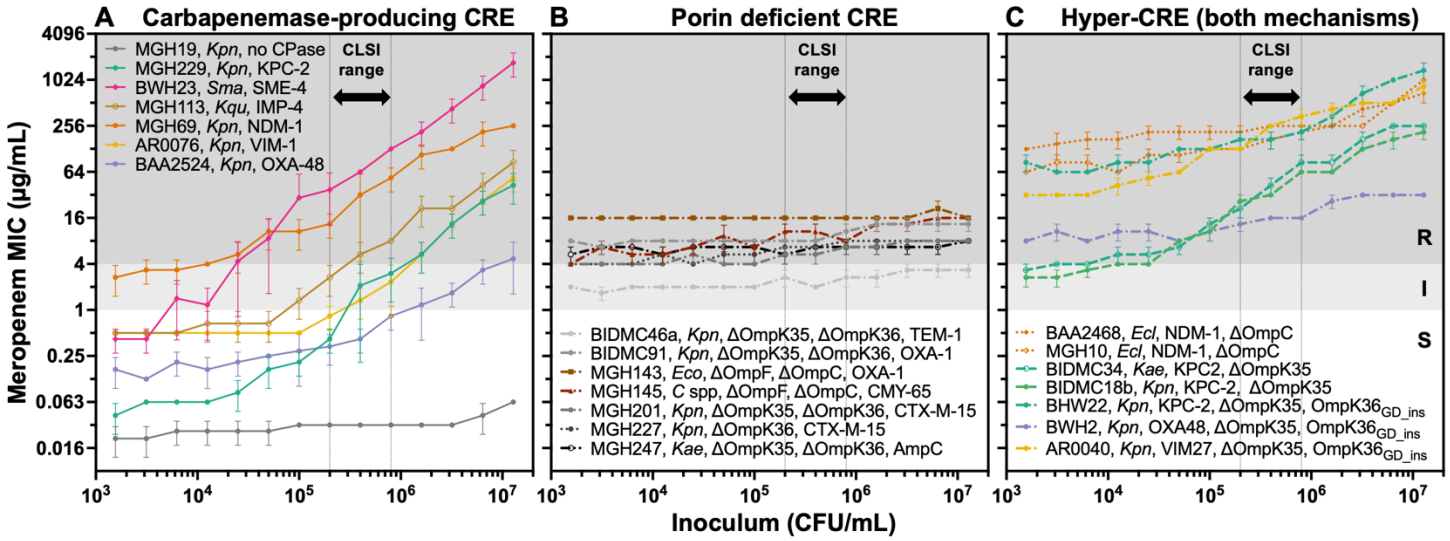

Fig. 2. Select CRE exhibit different patterns of carbapenem inoculum effect depending on resistance mechanism. Meropenem MICs from broth microdilution assays (y-axis) across a broad range of starting inocula (x-axis) of select clinical CRE isolates encoding **(A)** a carbapenemase of each major type, **(B)** porin deficiency, or **(C)** both, with species and relevant genotype as indicated. One susceptible isolate (MGH19, gray) is also shown in panel **(A)**. Each MIC point is the mean of three replicates (error bars = standard error of the mean). *C spp* = *Citrobacter* spp, *Ecl* = *Enterobacter cloacae*, *Eco* = *Escherichia coli*, *Kae* = *Klebsiella aerogenes*, *Kpn* = *Klebsiella pneumoniae*, *Kqu* = *Klebsiella quaispneumoniae*, *Sma* = *Serratia marcescens*. CPase = carbapenemase. Vertical dotted lines reflect the CLSI-recommended inoculum range, background shading reflects CLSI breakpoints, and line color reflects carbapenemase content, all as in Fig. 1; dashed lines indicate porin deficiency.

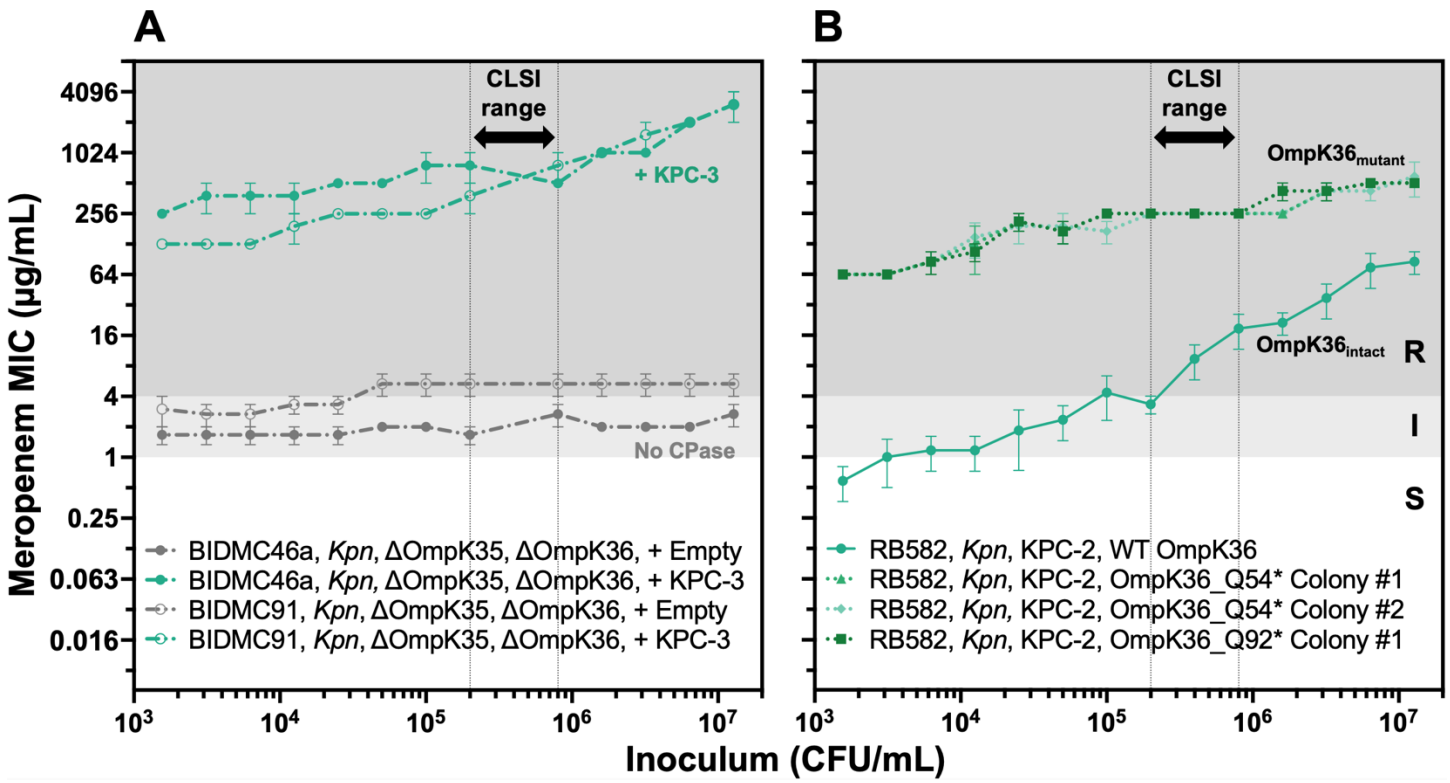

Fig. 3. Carbapenemase production and porin deficiency together create a hyper-CRE phenotype. Broad-range meropenem broth microdilution assays for **(A)** two clinical PD-CRE isolates before and after transformation with pBAD33_KPC-3 and **(B)** a clinical CP-CRE isolate and three derivative colonies in which OmpK36 was disrupted by CRISPR-Cas9 based gene editing to create a premature stop at one of two glutamines as indicated. The parental KPC-expressing isolate (RB582) is shown as a solid line with green markers. Each MIC point is the mean of three replicates (error bars = standard error of the mean). Vertical dotted lines reflect the CLSI-recommended inoculum range, background shading reflects CLSI breakpoints, and line color reflects carbapenemase content as in Fig. 1; dashed lines indicate porin deficiency as in Fig. 2. *Kpn* = *Klebsiella pneumoniae.*

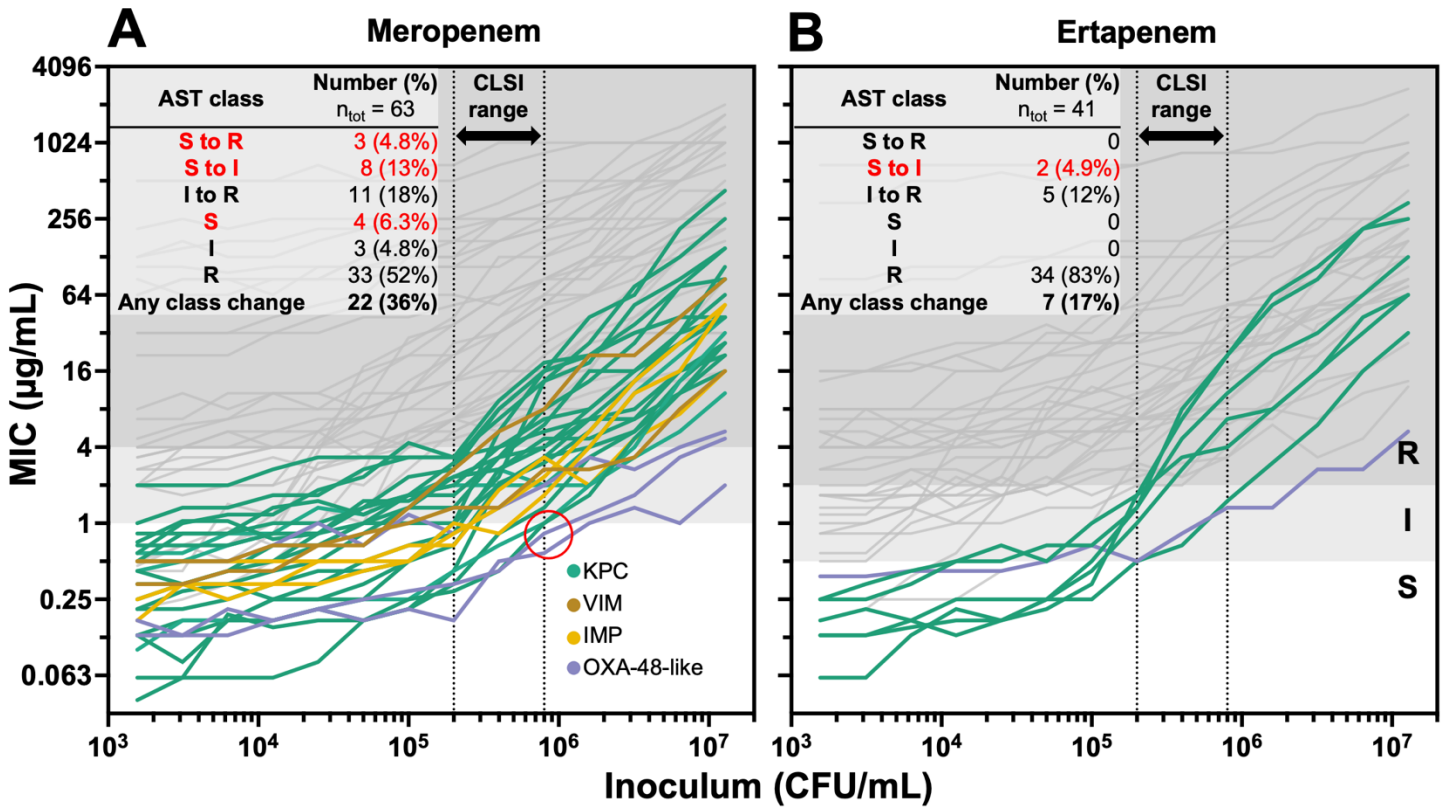

Fig 4. Many carbapenemase-producing isolates change AST classification in the CLSI range. Broad-range broth microdilution assays for **(A)** 63 carbapenemase-producing clinical CRE isolates (36 CP-CRE and 27 hyper-CRE) treated with meropenem and **(B)** 41 carbapenemase-producing clinical CRE isolates (30 CP-CRE and 11 hyper-CRE) treated with ertapenem. Each MIC measurement was the mean of three replicates. Error bars were omitted for simplicity. Isolates that tested anything other than resistant at some point in the CLSI-recommended inoculum range are colored by carbapenemase content; those that tested resistant throughout the CLSI range are in gray. Inset table shows the number and percentage of isolates by AST class across the CLSI inoculum range; red text indicates isolates that tested susceptible at some point within the CLSI range. Four CP-CRE that tested fully meropenem-susceptible throughout the CLSI range are indicated with a red circle.

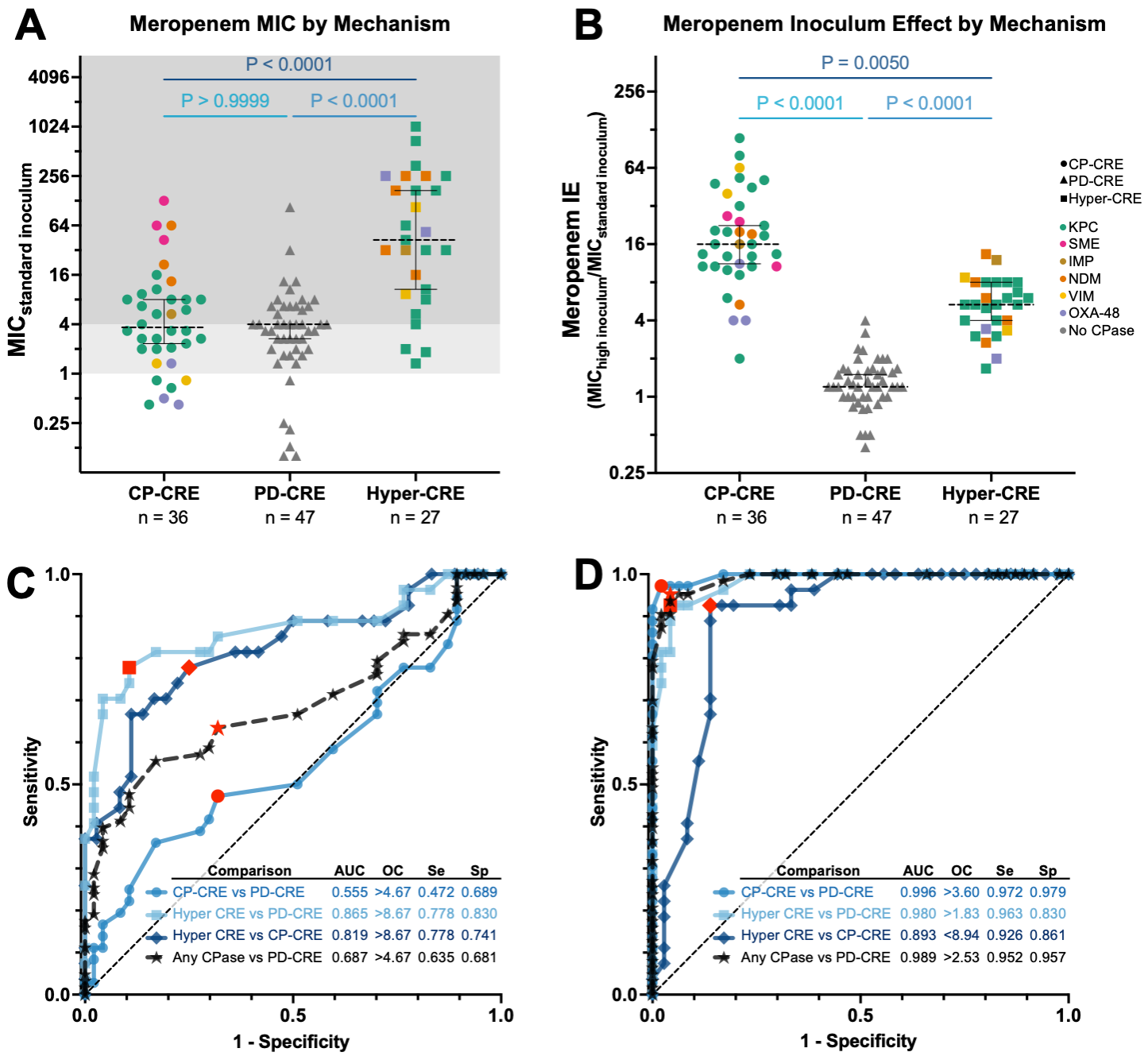

Fig. 5. Meropenem inoculum effect robustly classifies CRE isolates by resistance mechanism. (A) Meropenem MICs for 110 CRE grouped by genotypic mechanism of carbapenem resistance: CP-CRE (circles), PD-CRE (triangles), or hyper-CRE (squares). Isolates are colored by carbapenemase content as in Fig. 1. Each MIC point is the mean of three replicates. **(B)** Ratio of meropenem MIC at high (1.3 x 10^7 CFU/mL) versus standard (4.0 x 10^5 CFU/mL) inoculum for 110 CRE, grouped as in **(A).** P-values were obtained by conducting statistical analyses on the three groups in **(A)** and **(B)** using a Kruskal-Wallis test. **(C)** Receiver operating characteristic (ROC) curves based on the meropenem MICs at standard inoculum from **(A)** comparing the different genotypic CRE groups. Inset tabulates each ROC area under the curve (AUC), sensitivity (Se), and specificity (Sp) at an optimal cutoff (OC) threshold, indicated by the red points on the ROC curves. **(D)** ROC curves based on the meropenem inoculum effect, displa+yed as in **(C)**. Any CPase = either CP-CRE or hyper-CRE.

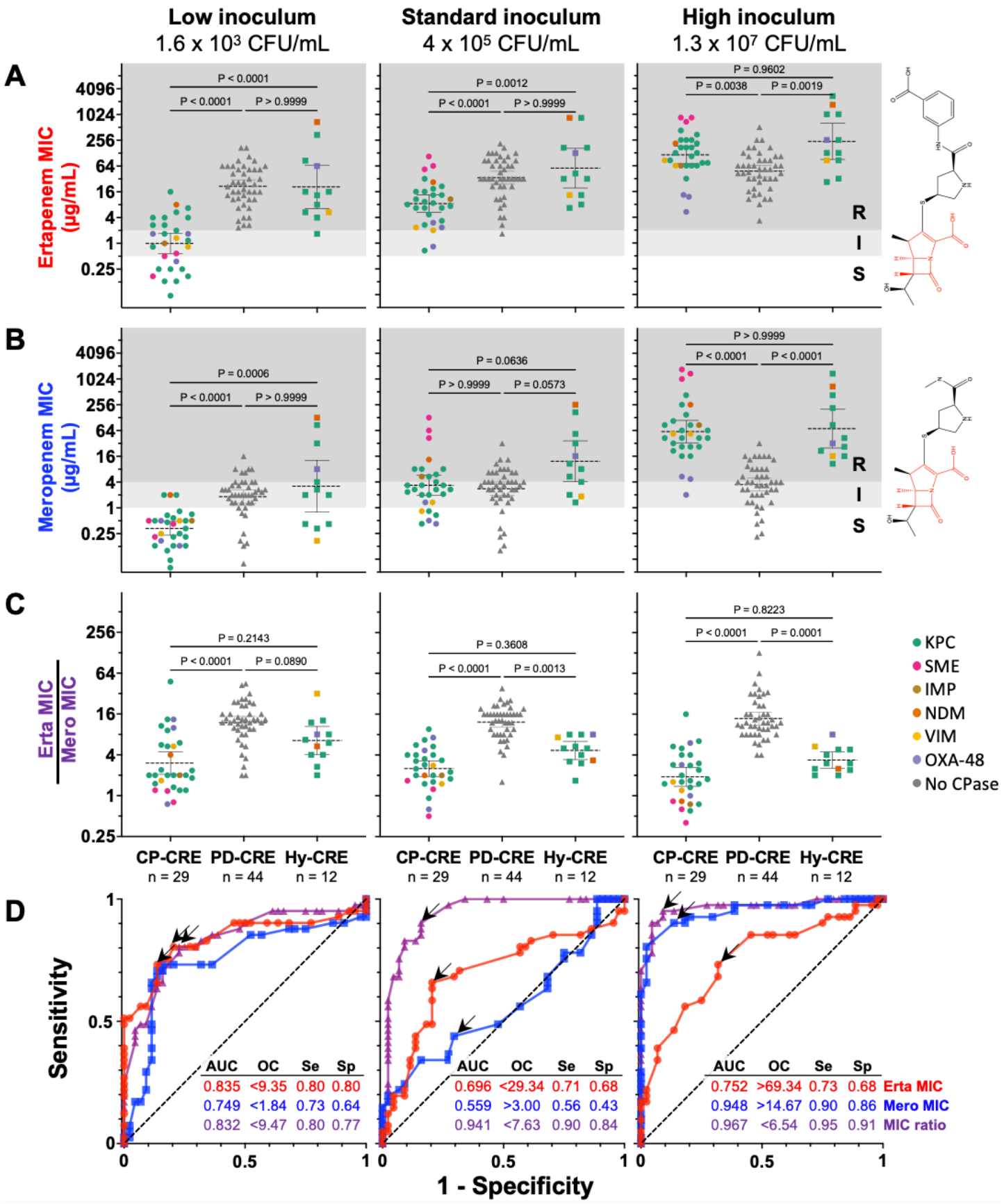

Fig. 6. Comparing ertapenem and meropenem MICs gives insight into resistance mechanism (A-C) MICs of ertapenem **(A)** or meropenem **(B)**, or the ratio of the ertapenem MIC to the meropenem MIC **(C),** are shown for 85 isolates at low, standard, and high inoculum (left, middle, and right panels respectively), grouped

by resistance mechanism along the x-axis (hyper-CRE = “Hy-CRE”). Data points are colored based on carbapenemase content as in Fig. 1. Plot backgrounds are shaded by CLSI breakpoints for ertapenem **(A**) or meropenem **(B**) as in Fig. 1. P-values were obtained by conducting statistical analyses on the three groups in using a Kruskal-Wallis test. In chemical structures, the carbapenem core is red and the modifiable substituents are black. **(D)** ROC curves comparing carbapenemase producers (CP-CRE + hyper-CRE) vs PD-CRE for each metric (red = ertapenem MIC, blue = meropenem MIC, purple = MIC ratio) at low, standard, and high inoculum. Inset tabulates ROC AUC and sensitivity (Se) and specificity (Sp) at an optimal cutoff (OC, indicated by arrow on ROC curve).