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 hydrolysis or reduced antibiotic influx. Identifying these mechanisms can improve pathogen surveillance, infection control, and patient care. We investigated how resistance mechanisms influence the carbapenem inoculum effect (IE), a phenomenon where inoculum size affects antimicrobial susceptibility testing (AST). We demonstrated that seven different carbapenemases impart a meropenem IE in *Escherichia coli*. Across 110 clinical CRE isolates, the carbapenem IE strictly depended on resistance mechanism: all carbapenemase-producing CRE (CP-CRE) exhibited a strong IE, whereas porin-deficient CRE displayed none. Concerningly, 50% and 24% of CP-CRE isolates changed susceptibility classification to meropenem and ertapenem, respectively, across the allowable inoculum range in clinical guidelines. The meropenem IE, and the ratio of ertapenem to meropenem minimal inhibitory concentration (MIC) at standard inoculum, reliably identified CP-CRE. Understanding how resistance mechanisms affect AST could improve diagnosis and guide therapies for CRE infections.

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 pathogens (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 carbapenemase activity such as AmpC, CTX-M, SHV, TEM, or OXA-10, and together cause resistance (1, 7). Efflux pumps can also contribute to carbapenem resistance, though their role has been better characterized in non-*Enterobacterales* species like *Acinetobacter baumannii* and *Pseudomonas aeruginosa* (8, 9). Carbapenemase-producing CRE (CP-CRE) are of particular epidemiological concern due to their propensity to cause outbreaks (4, 10) and the risk of horizontal transfer of carbapenemase genes (11–13). Porin deficient CRE (PD-CRE) often exhibit impaired fitness (14) compared to CP-CRE because porins play important roles in nutrient import (15), osmotic homeostasis (14, 16), and membrane lipid asymmetry (17). Determining resistance mechanisms could lead to better infection control measures, such as patient isolation to prevent CP-CRE spread in hospitals (4, 13, 18). Some laboratories test for resistance mechanisms in CRE (19–21), but this

requires additional time, equipment, expertise, and expenses. Capitalizing on existing antimicrobial susceptibility testing (AST) workflows to predict CRE resistance mechanisms would represent a clinical advance.

Standardized AST methods and interpretive criteria are crucial to determine antimicrobial susceptibility of pathogens. Organizations like the Clinical and Laboratory Standards Institute (CLSI) classify pathogen susceptibility based on minimum inhibitory concentration (MIC) breakpoints (22–24) that are established and periodically revised (25) based on microbiological, pharmacokinetic-pharmacodynamic, and clinical data. While these breakpoints are intended to predict clinical response to therapy, not resistance mechanism, carbapenem MICs have been repurposed to prioritize isolates for molecular carbapenemase testing (22, 26). In the United States, only isolates that test phenotypically carbapenem resistant are typically tested for carbapenemases (22). Yet despite recently updated breakpoints (27), some carbapenemase producing isolates can still be misclassified as susceptible to carbapenems (28).

The inoculum effect (IE) is a phenomenon whereby the measured MIC varies depending on the precise bacterial inoculum used in AST assays, which can lead to susceptibility misclassification and treatment failures (29, 30). Multiple factors may contribute to the IE, including metabolic state of the bacteria (31), growth productivity (32), antibiotic-target ratio (33), heteroresistance frequency (34), target proteolysis (35), and production of enzymes that inactivate antibiotics (36–40). The IE is frequently observed for some antibiotic classes including β-lactams (41) but not commonly for others such as aminoglycosides. While importance of the IE in clinical infections remains uncertain (42, 43), it has important implications for diagnostic accuracy. To minimize the impact of the IE, CLSI recommends a standard 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, the gold standard AST method (44). Even within this range, however,

carbapenem susceptibility classification of *Enterobacterales* isolates may change due to the IE, underscoring the need for continued efforts to optimize AST methods (45).

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 *Escherichia coli*. Further, in a collection of 110 clinical CRE isolates across 12 species of *Enterobacterales* (11, 46), we observed that the carbapenem IE was a function of resistance mechanism. 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. These findings suggested two phenotypic measures that predict carbapenem resistance mechanisms better than the MIC cutoffs currently used to prioritize molecular carbapenemase testing.

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 (47, 48). 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 (49): $bla_{\text{KPC-3}}$ 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; $bla_{\text{CMY-10}}$ (50) from class C; and $bla_{\text{OXA-48}}$ from class D

(Supplementary 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, all transformants showed a significant increase in MIC, while the MIC of the empty vector control did not (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, which includes the recommended inoculum range for clinical testing (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) conferred 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). 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 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 Supplementary 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 Supplementary 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 Supplementary Fig. S1*B*). Finally, the 27 isolates with both carbapenemase production and

150 porin deficiencies, which we termed “hyper-CRE”, exhibited an IE and higher-level resistance
151 than isolates containing only one mechanism of resistance (Fig. 2*C* and Supplementary Fig.
152 S1*C*).
153
154 **Carbapenemase production and porin deficiency together cause a hyper-CRE phenotype.**
155 To directly test the hypothesis that the combination of carbapenemase production and porin
156 deficiency confers a hyper-CRE phenotype, we selected two clinical *Klebsiella pneumoniae*
157 isolates (BIDMC46a and BIDMC91) with disruptions in both major porins OmpK36 and
158 OmpK35, and transformed them with a pBAD33 plasmid encoding a KPC-3 (Fig. 3*A*). Both
159 transformants exhibited dramatic increases in MIC at standard inoculum (256-fold and 128-fold,
160 respectively) compared to empty vector controls and also developed an IE (6-fold from standard
161 to high inoculum). In a second experiment, we disrupted the major OmpK36 porin in a *K.*
162 *pneumoniae* isolate expressing a KPC-2 (RB582) using a CRISPR-Cas9 base editor (51).
163 Meropenem MICs at standard inoculum increased by 32-fold in the edited strain, with a small 2-
164 fold IE (Fig. 3*B*). These experiments provide direct evidence that the combination of
165 carbapenemase production and porin deficiency leads to a hyper-CRE phenotype.
166
167 **A significant proportion of carbapenemase-producing isolates change classification**
168 **within the CLSI inoculum range.** Although clinical microbiological laboratories initially
169 identified the isolates we studied as carbapenem-resistant, the IE caused the MIC of several
170 isolates to vary greatly within the CLSI inoculum range (Fig. 2*A*, and Supplementary Fig. S1*A*).
171 This variability prompted us to quantify which carbapenemase-producing isolates changed
172 susceptibility classification within the acceptable CLSI inoculum range of 2×10^5 CFU/mL to $8 \times$
173 10^5 CFU/mL. We tested both meropenem and ertapenem, two carbapenems in common clinical
174 use with distinct physicochemical and pharmacokinetic properties. Notably, ertapenem has a
175 lower barrier to resistance (52) since it is more affected by reduced outer membrane

176 permeability (53), and has lower clinical susceptibility breakpoints (22). 22 of 63 (35%)
177 carbapenemase-producing isolates tested with meropenem and 7 of 41 (17%) tested with
178 ertapenem changed AST classification (Fig. 4). When excluding hyper-CRE and considering
179 only isolates with intact porins, 18 of 36 (50%) CP-CRE isolates changed AST classification for
180 meropenem and 7 of 29 (24%) for ertapenem within the CLSI inoculum range. Concerningly,
181 three isolates changed from meropenem susceptible to resistant within the CLSI range (Fig. 4,
182 dotted lines), including one *E. coli* isolate (BIDMC43b) and two *Serratia marcescens* isolates
183 (BWH56 and BWH57), each of which encodes a KPC-3. Four isolates tested fully susceptible to
184 meropenem throughout the CLSI inoculum range (red circle), including two *Citrobacter freundii*
185 harboring KPC-3 (MGH281 and MGH283), and one *E. coli* (BAA2523) and one *K. pneumoniae*
186 (BAA2524) each harboring an OXA-48. In all, 15 of 63 (24%) carbapenemase producers (CP-
187 CRE or hyper-CRE) tested meropenem susceptible, and 2 of 41 (4.9%) tested ertapenem
188 susceptible, at some point in the CLSI inoculum range; this represented 15 of 36 (42%) and 2 of
189 29 (6.9%) CP-CRE, respectively.
190
191 **The inoculum effect accurately identifies carbapenemase production among CRE**. The
192 meropenem MIC at standard inoculum poorly distinguished carbapenemase-producing isolates
193 (CP-CRE or hyper-CRE) from PD-CRE (Fig. 5*A*), making it an inefficient metric to trigger
194 carbapenemase testing. By contrast, the clear phenotypic differences across inocula between
195 these groups led us to investigate a diagnostic approach based on the IE. We quantified the IE
196 by dividing the MIC at high inoculum (1.3×10^7 CFU/mL) by the MIC at standard inoculum ($4 \times$
197 10^5 CFU/mL) for each isolate (Fig. 5*B*). CRE exhibited distinct meropenem IEs depending on
198 their mechanism of resistance, with carbapenemase-producing isolates (CP-CRE and hyper-
199 CRE) exhibiting a much more prominent IE, with a median increase in MIC of 10-fold, compared
200 to a 1.2-fold increase for PD-CRE (Fig. 5*B*). To quantify binary classification distinguishing
201 carbapenemase-producing isolates from porin-deficient ones, we generated receiver operating

202 characteristic (ROC) curves using meropenem MIC (Fig. 5C) or IE (Fig. 5D) threshold values.
203 The area under each ROC curve (AUC) represents an aggregate measure of classification
204 accuracy in distinguishing isolates by resistance mechanism across all possible binary
205 thresholds of the metric being plotted. The meropenem IE distinguished carbapenemase-
206 producing CRE from PD-CRE better than meropenem MIC at standard inoculum, illustrated by a
207 ROC AUC of 0.99 for the meropenem IE versus 0.69 for meropenem MIC. To efficiently identify
208 CRE that might carry carbapenemases, precise IE threshold values can be chosen to optimize
209 sensitivity (detecting carbapenemases) while maintaining reasonable specificity. At one specific
210 IE threshold of 2.53-fold, the sensitivity and specificity of distinguishing carbapenemase-
211 encoding isolates from PD-CRE was 95.2% and 95.7%, respectively. The meropenem IE was
212 greater in CP-CRE (median 16-fold MIC increase) than hyper-CRE (median 5.3-fold), and it also
213 distinguished these two groups (Fig. 5 *B* and *D*; ROC AUC 0.89).
214
215 **Comparing ertapenem and meropenem MICs gives insights into resistance mechanisms.**
216 The meropenem IE effectively distinguished carbapenemase-producing isolates from PD-CRE,
217 but it is not a standard workflow in clinical laboratories, which typically only perform AST at
218 standard inocula. To overcome this limitation, since the periplasmic permeability of ertapenem is
219 typically more impacted by porin mutations than that of meropenem (6, 7, 44, 54), we
220 hypothesized that comparing ertapenem and meropenem MICs, even at a single inoculum,
221 might also distinguish CRE by mechanism of resistance. We thus measured ertapenem MICs
222 across a broad inoculum range for 85 of the 110 CRE isolates (Fig. 6). The ratio of ertapenem
223 MIC to meropenem MIC better separated carbapenemase producing isolates (CP-CRE or
224 hyper-CRE) from PD-CRE (Fig. 6*C*) than either the ertapenem or meropenem MIC alone (Fig. 6
225 *A* and *B*). At standard inoculum, meropenem MIC performed poorly at distinguishing
226 carbapenemase producers (CP-CRE or hyper-CRE) from PD-CRE (ROC AUC 0.559), whereas
227 ertapenem MIC performed slightly better (ROC AUC 0.696; Fig. 6*D*). By contrast, the

ertapenem-to-meropenem MIC ratio yielded a ROC 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 *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, Supplementary 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, all carbapenemases imparted a strong inoculum effect despite minimal sequence or structural similarity across the four Ambler classes. Furthermore, we observed a consistently biphasic dependence of CP-CRE MIC on inoculum (Fig. 1, Fig. 2 and Supplementary Fig. S1): at the lowest inocula tested, carbapenemases conferred a relatively constant degree of resistance, whereas above a certain threshold, resistance of the population increased with cell density, by up to 100-fold at the highest cell densities compared with the standard inoculum (Fig. 1B, Fig. 2A). This is consistent with a model where carbapenemases provide cell-autonomous resistance at low density but increasing population-level resistance as cell density increases, such that the collective production of carbapenemases may become the

dominant determinant of resistance at high enough cell density. Since the IE is only observed with carbapenemase producers and not PD-CRE, one intriguing possibility is that carbapenemases serve as a "common good" in bacterial populations above a certain density threshold, as has been proposed for other secreted enzymes (36–38, 55). One recent study of bacterial communities in which some members produced OXA-48 supports this model (40), but further studies with mixed populations of carbapenemase producing and non-producing isolates are needed to rigorously test this hypothesis. 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 Supplementary Fig. S1*B*), indicating that the slow rates of carbapenem hydrolysis by ESBL enzymes were insufficient to benefit neighboring bacteria even at high culture density. The lower prevalence of carbapenemases compared to other β-lactamases may explain why the IE is reported less frequently for carbapenems than for other β-lactams (56), but this might change as carbapenemases continue to spread globally. The extent of and mechanistic basis for carbapenemase release into the extracellular space where they may 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 most infections *in vivo* are generally below those used for AST (45, 55). 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 (57, 58). Nevertheless, in *Staphylococcus aureus* bacteremia, a cefazolin IE has been associated with treatment failure and higher mortality (59, 60). Our findings suggest that at the low inoculum present in bacteremia, PD-CRE (Fig. 2*B* and Supplementary 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 bacteremia (61, 62). It is possible that the increased resistance of PD-CRE at physiologically relevant bacterial 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. Intriguing recent studies found that isolates encoding carbapenemases and displaying a large inoculum effect require higher meropenem concentration to prevent emergence of highly resistant mutants (39, 52). Since we observed the largest IE among CP-CRE with intact porins, perhaps these extremely resistant escape mutants reflect *de novo* porin mutations, creating hyper-CRE.

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 (45), particularly impacting the detection of CP-CRE, the isolates that carry the greatest implications for infection control. Even though the 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. In fact, MICs of all CP-CRE isolates varied significantly in this range. Our observations in *E. coli* K-12 (Fig. 1) reveal that strains harboring OXA-48, CMY-10, or even KPC might only test resistant if they possess other factors, such as reduced antibiotic permeability. 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 (22). 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. Although MICs

were not designed to identify resistance mechanisms, carbapenem MICs are routinely used to prioritize isolates for molecular carbapenemase testing (63), though different MIC thresholds for testing are recommended in the US (22) and Europe (26). 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). 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, 44, 54), while both are hydrolyzed at similar rates by carbapenemases (64, 65). Predicting porin function has been a major challenge for genomic resistance prediction (66–71), as polymorphisms are frequently found but challenging to functionally interpret. Given the differential impact of porin mutations on each carbapenem, the ertapenem-to-meropenem MIC ratio may offer a phenotypic reflection of porin function. 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 resistance mechanism independent of inoculum, an advantage over either ertapenem or meropenem MICs alone (Fig. 6).

We propose that this ertapenem to meropenem MIC ratio might therefore be better suited than either MIC alone to inform downstream testing and prioritize isolates for molecular or enzymatic carbapenemase detection (19, 72, 73). Recognizing that MIC thresholds for susceptibility may miss carbapenemase-producing isolates, EUCAST recommends a 16-fold lower MIC threshold for carbapenemase screening than their clinical susceptibility breakpoint (26). This lower threshold will lead to more sensitive carbapenemase detection, but at lower

specificity, requiring far more testing. If validated in further studies at this lower MIC threshold, the ertapenem to meropenem MIC ratio may enable optimization of strain selection for molecular carbapenemase testing. By more efficiently identifying carbapenemase-producing isolates, our work could facilitate implementation of recent advances showing that molecular detection of carbapenemases can accelerate administration of targeted therapies, such as β-lactamase inhibitor combinations (74), and decrease mortality (21). Moreover, this phenotypic approach could detect strains producing rare carbapenemases, such as SME or CMY-10, that are not routinely included in testing panels, akin to the Carba NP (22, 75) and mCIM methods (20). This finding, in addition to its therapeutic value, could serve as a surveillance tool for the emergence and evolution of carbapenemases (76). This work does not aim to replace well-validated clinical carbapenemase detection methods. Instead, it illustrates how biological phenomena can offer further insights into resistance mechanism in a clinical context.

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 these lower-resolution MIC estimates in screening for CP-CRE. Third, while we found carbapenemases 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 requiring other factors. Our findings reveal a clear pattern: as the inoculum increases above a certain threshold, we observe a steady linear rise in MIC (Fig. 2 and Supplementary Fig. S1). This pattern is less consistent with heteroresistance, an alternative explanation for the IE whereby a

rare resistant subpopulation emerges from larger inocula. With heteroresistance, one would expect a sudden step-like increase in MIC as inoculum rises, as opposed to the gradual MIC increase that we consistently observed. Lastly, this study did not explore the basis of 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 one major mechanism underlying the IE. Future studies could involve pairing these detailed phenotypic data and genotypic information (*SI Appendix*, Table S3) with precise enzymological parameters and direct quantification of enzymes and metabolites in different compartments in order to enhance and experimentally test existing quantitative models (37, 55). 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.

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*$_{KPC-3}$, *bla*$_{SME-2}$, *bla*$_{IMP-4}$, *bla*$_{NDM-1}$, *bla*$_{VIM-27}$, and *bla*$_{OXA-48}$ along with their native promoters were cloned by Gibson assembly from isolates in our existing CRE collection (11, 12) encoding these carbapenemases into a pBAD33 plasmid which encodes chloramphenicol resistance (*SI Appendix*, Table S1). *bla*$_{CMY-10}$ with its native promoter was synthesized in the same pBAD33 vector backbone by GeneWiz (Suzhou, China), since we did not have a clinical isolate expressing this enzyme.

Broad-range broth microdilution. Our MIC assays were adapted from previously published broth microdilution methods(24, 77). 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 Supplementary 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(51) was used to introduce an early termination codon in the intact *ompK36* of isolate RB582. See Supplemental Methods for further details.

410

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Figures

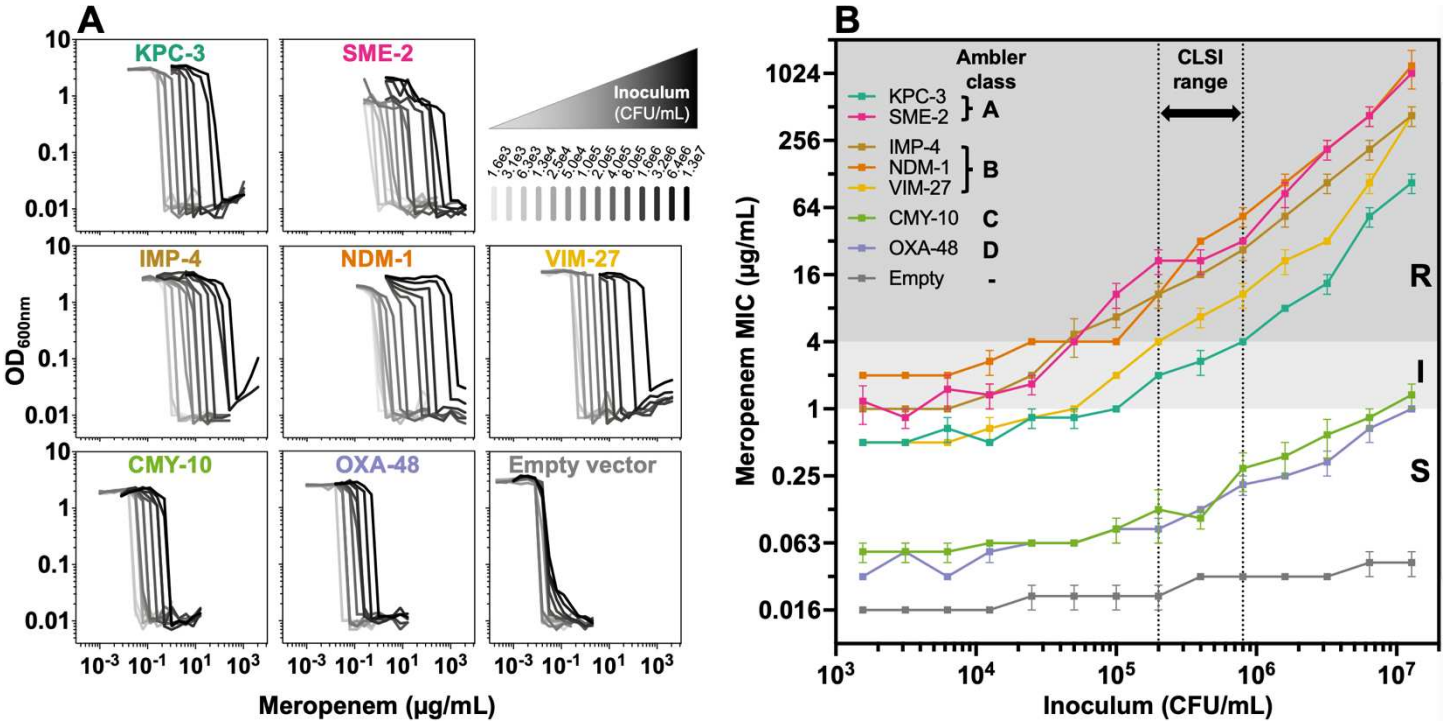

Fig. 1. All carbapenemases impart a meropenem inoculum effect when expressed in *E. coli K12*. (A) Plots of optical density (OD) at 600 nm (y-axis) after overnight incubation with varying meropenem concentrations (x-axis) across a broad range of starting inocula (grayscale per top right panel) for *E. 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).

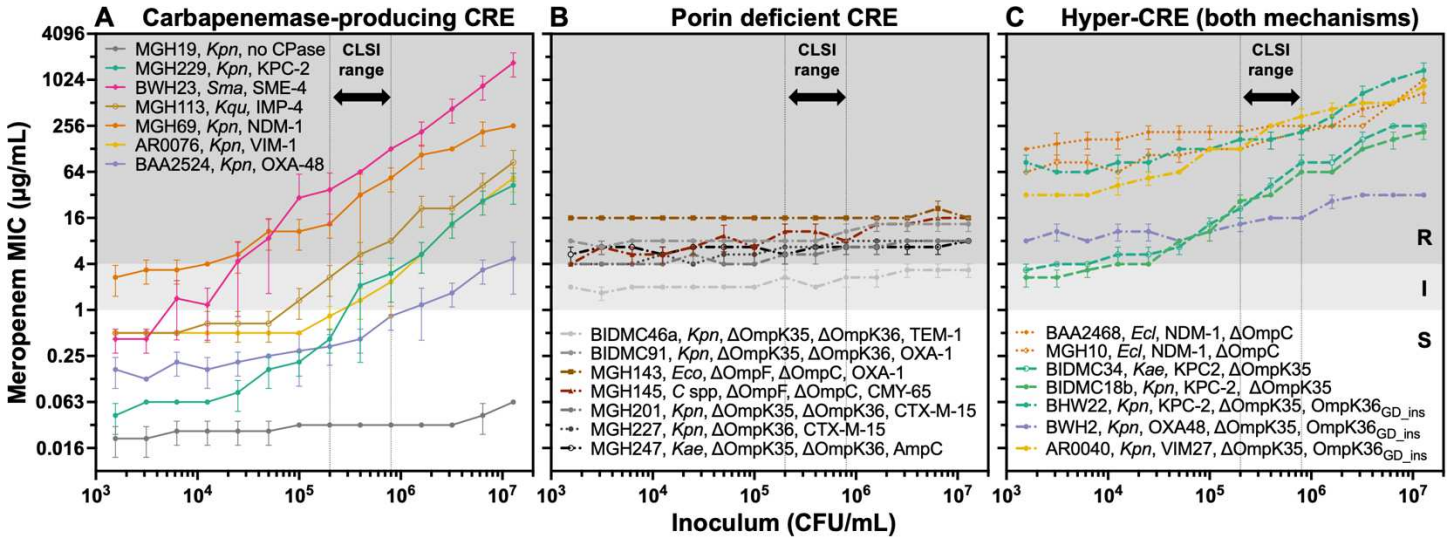

Fig. 2. Select CRE exhibit different patterns of carbapenem inoculum effect depending on resistance mechanism. Meropenem MICs from broth microdilution assays (y-axis) across a broad range of starting inocula (x-axis) of select clinical CRE isolates encoding **(A)** a carbapenemase of each major type, **(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.

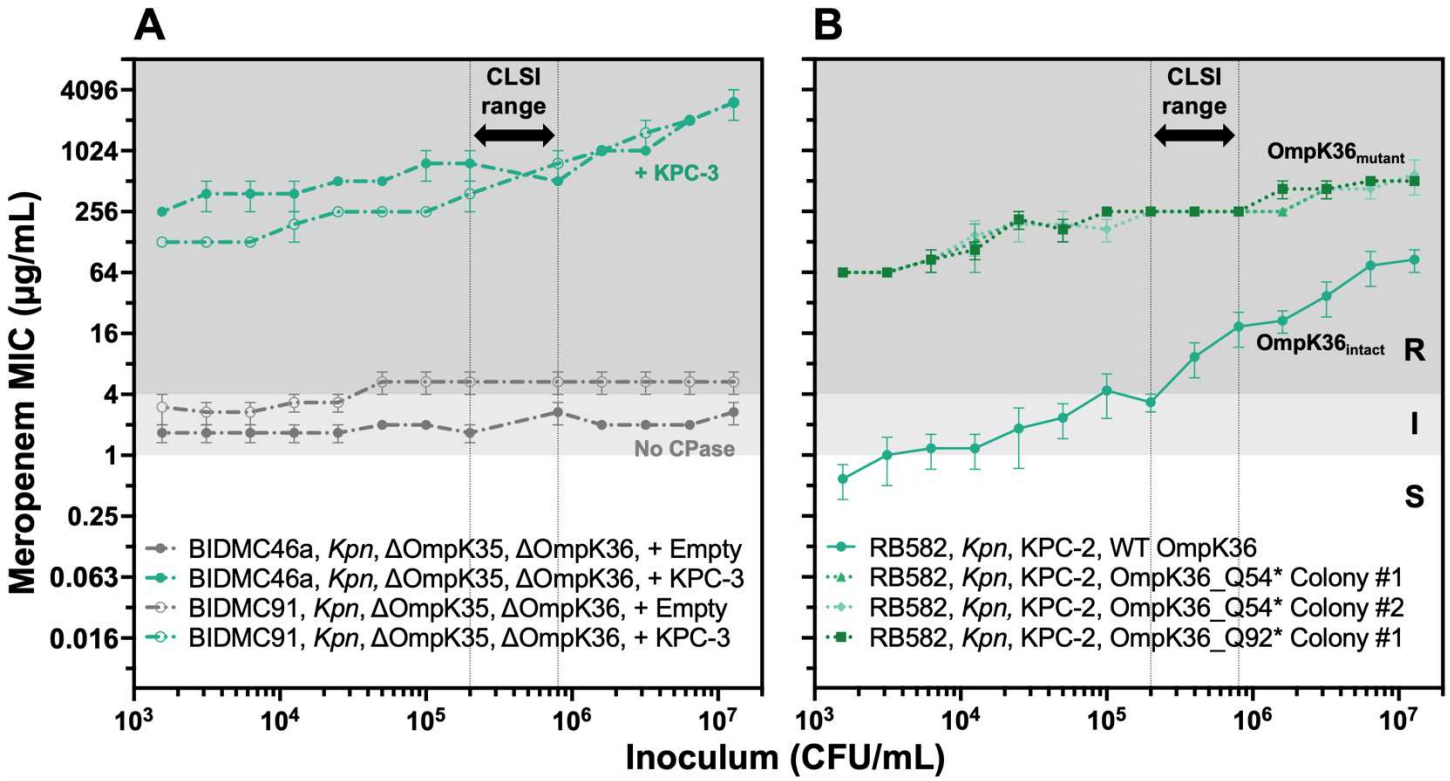

Fig. 3. Carbapenemase production and porin deficiency together create a hyper-CRE phenotype. Broad-range meropenem broth microdilution assays for **(A)** two clinical PD-CRE isolates before and after transformation with pBAD33_KPC-3 and **(B)** a clinical CP-CRE isolate and three derivative colonies 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.*

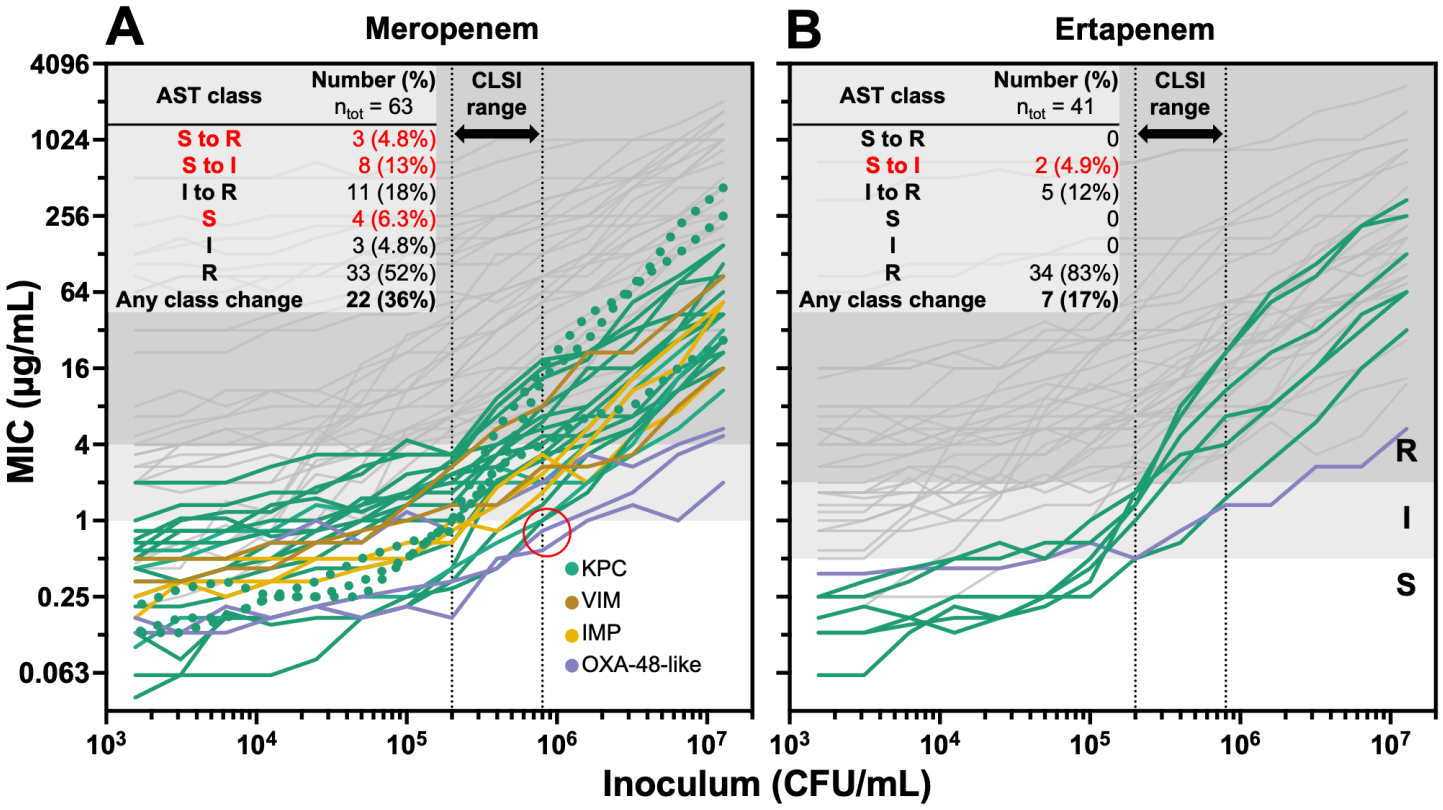

Fig 4. Many carbapenemase-producing isolates change AST classification in the CLSI range. Broad-range broth microdilution assays for **(A)** 63 carbapenemase-producing clinical CRE isolates (36 CP-CRE and 27 hyper-CRE) treated with meropenem and **(B)** 41 carbapenemase-producing clinical CRE isolates (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. Isolates whose meropenem susceptibility category changed from susceptible to resistant in the CLSI range are shown as dotted lines.

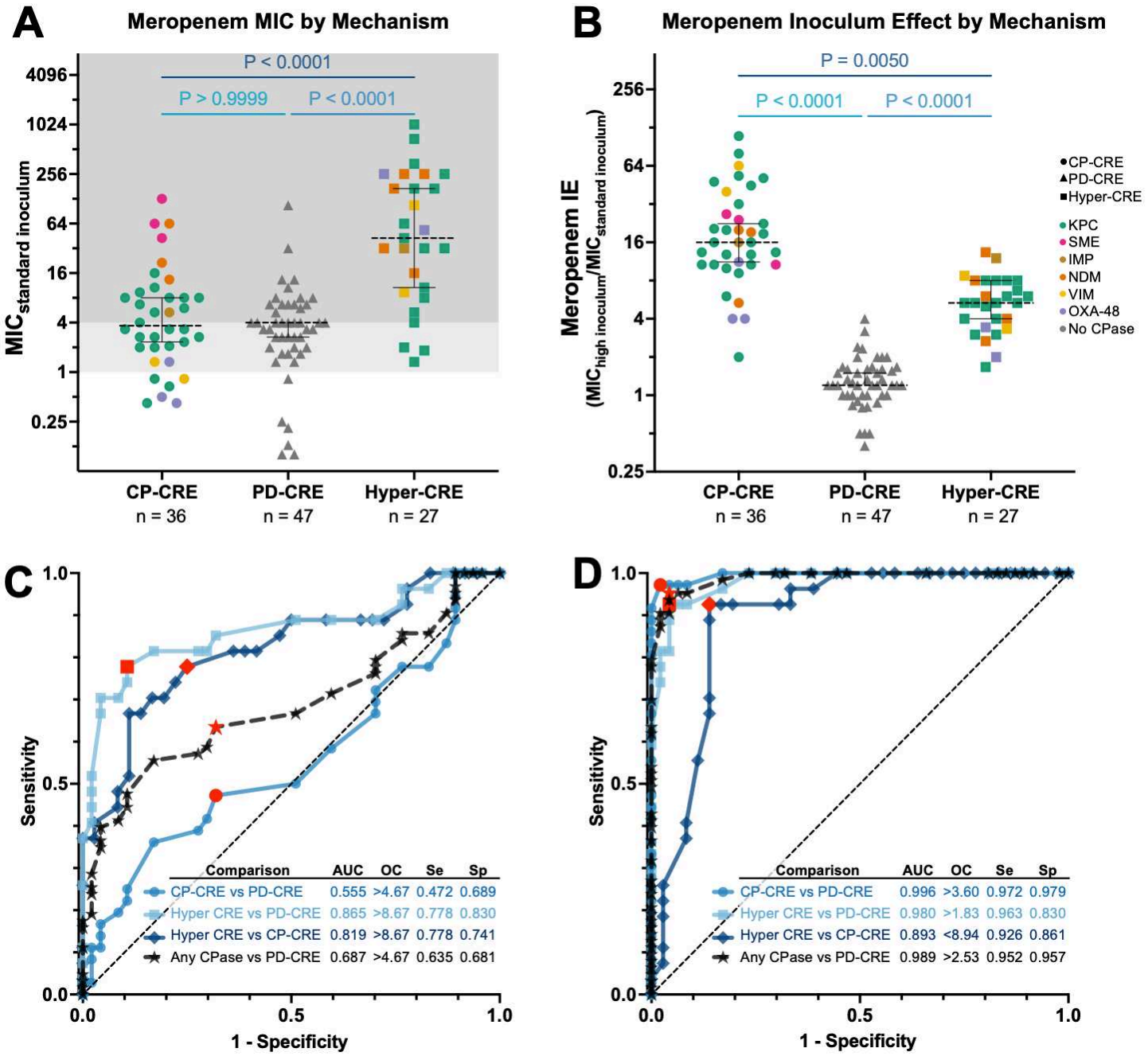

Fig. 5. Meropenem inoculum effect robustly classifies CRE isolates by resistance mechanism. (A) Meropenem MICs for 110 CRE grouped by genotypic mechanism of carbapenem resistance: CP-CRE (circles), PD-CRE (triangles), or hyper-CRE (squares). Isolates are colored by carbapenemase content as in Fig. 1. Each MIC point is the mean of three replicates. **(B)** Ratio of meropenem MIC at high (1.3×10^7 CFU/mL) versus standard (4.0×10^5 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, displayed as in **(C)**. Any CPase = either CP-CRE or hyper-CRE.

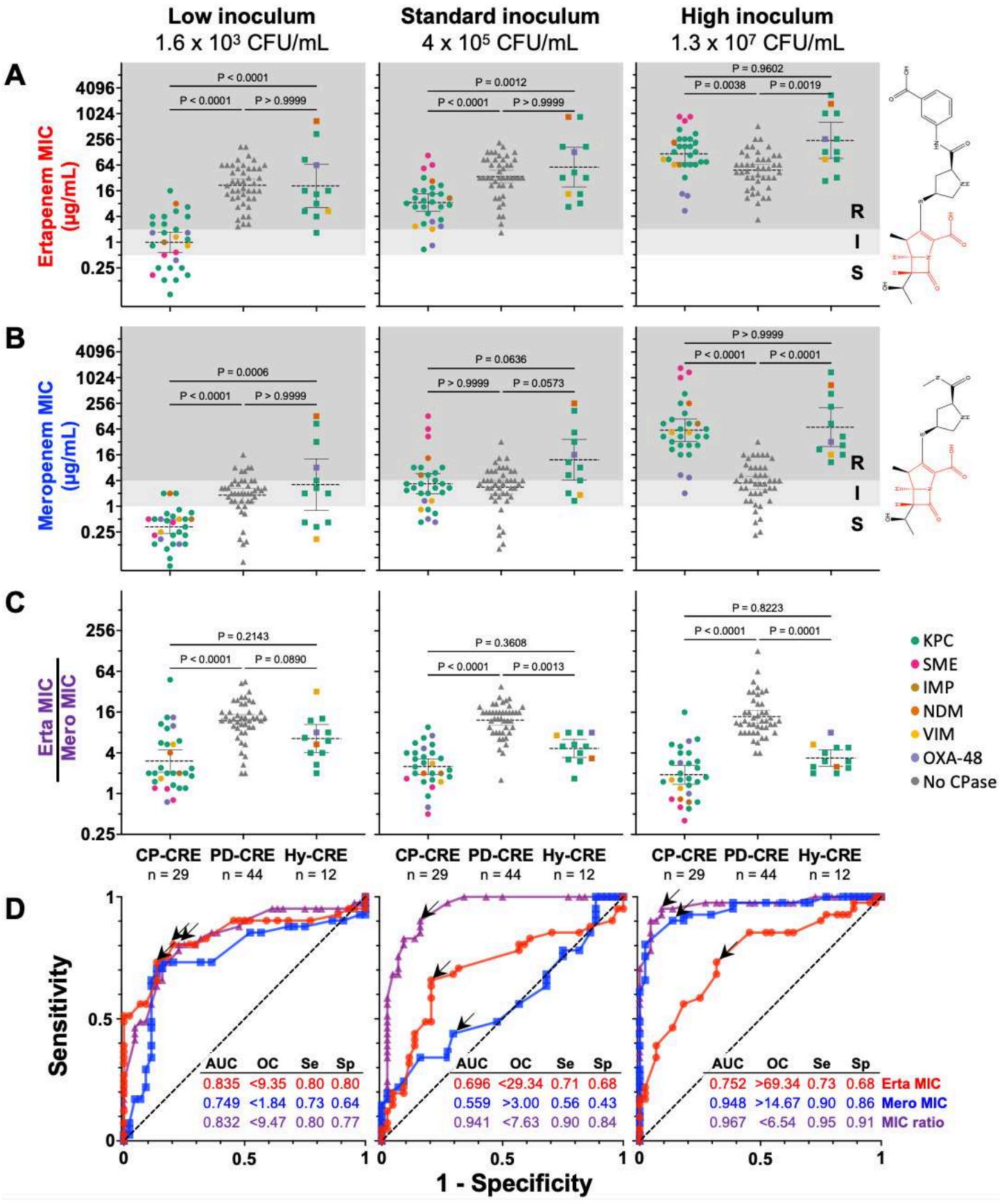

Fig. 6. Comparing ertapenem and meropenem MICs gives insight into resistance mechanism (A-C) MICs of ertapenem **(A)** or meropenem **(B)**, or the ratio of the ertapenem MIC to the meropenem MIC **(C),** are shown for 85 isolates at low, standard, and high inoculum (left, middle, and right panels respectively),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