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Characteristics and coordinated mechanisms of carbapenem heteroresistance in KPC-producing Enterobacteriaceae

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Characteristics and coordinated mechanisms of carbapenem heteroresistance in KPC-producing Enterobacteriaceae

by

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ABSTRACT

Characteristics and coordinated mechanisms of carbapenem heteroresistance

in KPC-producing Enterobacteriaceae

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Professor Lee W. Riley, Chair

Enterobacteriaceae strains producing the *Klebsiella pneumoniae* carbapenemase (KPC) have disseminated worldwide, causing an urgent threat to public health. KPC-producing strains often exhibit low-level carbapenem resistance, which may be missed by automated clinical detection systems. In these studies, eight *Klebsiella pneumoniae* strains, one *Enterobacter cloacae* strain and one *Escherichia coli* strain with heterogeneous resistance to imipenem were used to elucidate the factors leading from imipenem susceptibility (1-2ug/ml) to high-level resistance (>32ug/ml) as determined by clinical laboratory testing standards. These strains had highly similar heteroresistance phenotypes, yet were genetically diverse in their plasmid content other than carriage of the bla_{KPC}-containing Tn*4401*.

We showed that the heteroresistant strains had effective KPC-mediated inactivation of low levels of imipenem, combined with reduced or absent expression of *ompK35*, but as the imipenem concentration increased to 8- and 16-fold higher than the MIC, a majority (>99%) of the population was killed. Time-kill analysis with an inoculum as low as $3x10^6$ cfu/ml showed that full recovery of the population occurred by 20 hours of incubation in the same drug concentrations. We showed that it was not the density of the cultures per se, but cells with a distinct physiology, present at a frequency of $2x10^{-7}$ to $3x10^{-6}$ in starting cultures, and selected by lethal concentrations of imipenem, that coordinate population recovery subsequent to the killing of the majority of the initial cells. Samples selected 2 hours after exposure to imipenem were as susceptible as the unexposed parental strain and produced the major outer membrane porin OmpK36. However, between 4 to 8 hours after exposure, OmpK36 became absent and the imipenem MIC increased at least 32-fold. Individual colonies isolated from cultures after 20 hours of exposure revealed both susceptible and resistant subpopulations. We hypothesize that these heterogeneous populations arose from the small population of cells that initially survived imipenem-mediated killing.

There were two types of OmpK36 production among the *K. pneumoniae* heteroresistant strains. The majority (6 of 8) of the strains permanently abolished OmpK36 upon lethal imipenem exposure due to mobile insertion element interruptions in the coding region of *ompK36*. High-level imipenem resistance was maintained and OmpK36 remained absent even without continued carbapenem exposure. Two strains reverted to the heteroresistance phenotype and resumed production of OmpK36 once imipenem exposure was removed.

Through transposon mutagenesis of a reverter-type heteroresistant *K. pneumoniae* strain, we showed that acquisition of specific nutrients was essential to abolish OmpK36 production and for population recovery. Addition of the KPC enzyme inhibitor phenylboronic acid (PBA) at any point during imipenem exposure prior to loss of OmpK36 inhibited subsequent OmpK36 loss and prevented population recovery, showing the essentiality of bla_{KPC} in the expression of heteroresistance.

These studies demonstrated the coordination between bacterial physiology, bla_{KPC} and *ompK36* expression that led to the rapid induction of high-level imipenem resistance from a population of bacteria that initially exhibited a carbapenem-susceptibility phenotype. The finding of specific nutritional requirements for full expression of heteroresistance highlights potential therapeutic avenues of exploration for these strains that pose an urgent and increasing threat to public health.

For my husband, Neal Sapper, who was the first to suggest this was possible, removed many of the obstacles to make this possible – who never doubted it would come together– and who dealt graciously with the fallout when I did doubt.

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For Catherine Jacinta de la Cruz, "Aunt Kitty", who taught me to cherish life-long learning, and that an age-determined life-course is an artificial construct.

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CHAPTER ONE. Rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing *Klebsiella pneumoniae* (1).

INTRODUCTION

The widespread dissemination of carbapenem resistant Enterobacteriaceae (CRE) has reached a state of urgency in the US and abroad, greatly diminishing the ability to rely on carbapenems as the drug of last resort to treat multidrug-resistant CRE infections (2, 3). Strains that produce *Klebsiella pneumoniae* carbapenemase (KPC), encoded by the bla_{KPC} gene, first emerged with large-scale outbreaks in US hospitals, and are now one of the most important contributors to carbapenem resistance worldwide among Gram-negative bacteria (GNB) (3-7). KPC-producing strains co-harbor numerous drug-resistance determinants making clinical management of infections caused by such strains very complicated. Mortality exceeds 40% in patients infected with KPC-producing strains especially when the infection results in bacteremia (3, 5, 8-12). Failure to detect carbapenem resistance in a timely manner is a major contributor to the high rates of mortality in infections caused by KPC-producing GNB strains. Indeed, strains that harbor bla_{KPC} commonly exhibit low level resistance to carbapenem drugs, and are frequently missed due to inconsistency across various automated detection systems (8, 12-17). Moreover, carbapenemase-producing organisms are often detected only after patients fail therapy (11, 15). These strains often exhibit full or reduced susceptibility to a carbapenem according to standard laboratory testing (1-2ug/ml), but, upon single exposure to a carbapenem, generate subpopulations with MICs greater than 64ug/ml. Such strains are said to exhibit heteroresistance. Factors that determine carbapenem heteroresistance are unknown. Here we show how such conversion occurs through coordinated expression of bla_{KPC} and decreased production of the major outer membrane porin OmpK36.

METHODS

Strains and susceptibility testing. Antimicrobial susceptibility testing was performed by broth microdilution in accordance with the standards set by Clinical and Laboratory Standards Institute (CLSI) and ETEST® (bioMérieux, Marcy l'Etoile, France). Imipenem (Sigma-Aldrich, St. Louis, MO) was used as the representative carbapenem drug in all experiments. Phenylboronic acid (PBA, Sigma-Aldrich), an inhibitor of KPC hydrolysis,was used to analyze its effect on imipenem MICs. *K. pneumoniae* strains were obtained from rectal swabs, bloodstream and urinary tract infections collected by hospitals in Brazil and San Francisco. Eight KPC-producing *K. pneumoniae* strains with clinically relevant imipenem heteroresistant phenotypes, and three KPC-producing *K. pneumoniae* strains with high-level imipenem resistance were chosen from this set for our analysis (Table 1). Four non-KPC-producing *K. pneumoniae* clinical strains were chosen as controls. The KPC-producing strains belonged to three different multilocus sequence type (MLST) clonal groups. Strains were considered heteroresistant if colonies grew within the zone of inhibition with imipenem ETEST®. Heteroresistant strains were considered clinically relevant if their reference standard broth microdilution imipenem MIC was ≤2ug/ml. Preparation for all experiments was performed with one isolated colony from a freshly streaked

Mueller Hinton (MH) agar plate, which was grown overnight in MH broth at 37°C with shaking. Samples were tested in triplicate and experiments were performed at least three times.

Inoculum effect analysis. Inoculum-dependent increases in the MIC for imipenem were determined based on the CLSI reference standard starting inoculum of $5x10^5$ cfu/ml compared to inocula of $5x10^6$, $5x10^7$ and $5x10^8$cfu/ml. An inoculum effect was considered positive if the higher test inocula resulted in an 8-fold or greater increase in the imipenem MIC.

Population analysis. Population analysis was performed with 10^6 and 10^7 bacterial cfu spread on imipenem-containing MH agar plates (0.25-64ug/ml). We calculated the frequency of heteroresistant subpopulations at the highest drug concentrations after 24 hours of growth by dividing the number of colonies grown on imipenem-containing plates by the colony counts from the same bacterial inoculum plated onto drug-free MH agar plates (18).

Time-kill analysis. The frequency of survival in bactericidal concentrations of imipenem was quantified with starting inocula of $5x10^5$ and 1 to $9x10^6$ cfu/ml in a total volume of 3ml of MH broth, and with concentrations of imipenem 4- to 16-fold above the reference MIC. The starting inoculum was prepared from appropriate dilutions of overnight cultures standardized by optical density at 600nm (OD_{600}). Starting inocula were enumerated on drug-free agar plates. At 2, 4, 6, 8 and 20 hours after imipenem exposure, 50ul aliquots were serially diluted in 0.85% saline and plated on drug-free agar for enumeration. Control samples of the strain were grown in MH broth without drug and enumerated at the same time points. Population recovery was considered achieved if after 20 hours of drug exposure, enumeration yielded at least 10^9 cfu/ml, or if the OD_{600} of the cultures was greater than 1. The 20-hour end point was determined based on results of imipenem stability experiments (described below). Aliquots removed from the wells at 2, 4, 6, 8 and 20 hours after imipenem exposure were also plated on MH agar containing the same concentration of imipenem used in the time-kill analysis.

Bioassay for imipenem hydrolysis. Inocula of $5x10^5$ and $5x10^6$cfu/ml of heteroresistant KPC-producing strain were incubated in the same imipenem concentration used in time-kill experiments for 2, 8 and 20 hours. Triplicate samples were used for each time point. At each time point, the cells were spun down and the supernatant was passed through a 0.2um filter and frozen at -80°C. Aliquots were plated on LB agar to ensure they were cell-free. *E. coli* ATCC25922 reference strain was then used to test residual imipenem concentrations in these filtrates. Spontaneous imipenem hydrolysis was assessed by incubation of MH broth with the appropriate concentrations of imipenem for 4, 6, 12, 18 and 24 hours. The *E. coli* ATCC25922 reference strain was then inoculated into tubes of these preparations to perform standard imipenem broth microdilution testing. Fresh imipenem in MH broth was prepared as control.

PCR and sequencing of *bla*$_{KPC}$ structural region and outer membrane porin genes. We conducted PCR analysis of the Tn*4401* regions up and downstream of the *bla*$_{KPC}$ open reading frame with primers based on a report by Naas et al. (19), and with primers designed within this study by Primer-BLAST (NCBI) (Table 2). PCR analysis of the coding region of *ompK35* and *ompK36* was performed with primers designed by Primer-BLAST (NCBI). Sequencing was performed on an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA) at the University of California Berkeley DNA Sequencing Facility. We visually inspected,

edited and assembled the DNA sequences with BioEdit version 7.0.1, then used ClustalW to perform multiple alignment analyses of the sequences. Sequences were analyzed for single nucleotide polymorphisms (SNPs) between time kill survivor strains and unexposed parental strains. Sequences were compared to Tn*4401* structural genes, *ompK35*, *ompK36*, and *ompK37* genes deposited in the National Center for Biotechnology Information (NCBI) database by an updated version of the BLAST program.

Real-time reverse transcription PCR (RT-PCR) analysis. We performed real-time RT-PCR of bla_{KPC} gene expression for time kill survivor samples of four heteroresistant *K. pneumoniae* strains (BR6, BR7, BR14, BR21) according to previously published protocols with modifications for comparative quantification by the standard curve method (20). Expression was compared between imipenem unexposed samples and those exposed for 2, 4, 6, 8 or 20 hours. The *rpoB* gene was used as an endogenous reference. An untreated wild type sample of each strain was used as a calibrator gene standard. Total RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA) at each of the experimental time points. cDNA was generated by reverse transcription with random hexamer primers and Superscript III according to manufacturer's instructions (Life Technologies/Thermo Fisher Scientific, Waltham, MA). Samples were prepared with Maxima SYBR Green/Rox qPCR Master Mix (Thermo Fisher Scientific, Waltham, MA), and performed on an AB7300 Real Time PCR System (Applied Biosystems, Foster City, CA). All samples were amplified in triplicate. Comparative quantification (fold-change) of gene expression between samples was analyzed with the equation $2^{-\Delta\Delta CT}$, where $\Delta\Delta C_T = \Delta C_{T\,kpc} - \Delta C_{T\,rpoB}$.

Analysis of outer membrane proteins. Outer membrane proteins were isolated according to the method of Carlone et al. (21). Briefly, samples were grown in nutrient broth or MH broth at OD_{600} of 0.6, centrifuged at 5000xg for 10 minutes, washed and resuspended in 10mM HEPES buffer, pH 7.4 and sonicated. The sodium N-lauroyl sarcosinate insoluble outer membrane porins were selectively obtained by incubation in 10mM HEPES buffer with 2% sarkosyl, followed by 30 minute centrifugation at 15,600xg. Samples were boiled and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gels (Bio-Rad, Hercules, CA). Controls included drug-susceptible *Klebsiella pneumoniae* strains.

ESI-MS. Electrospray ionization mass spectrometry (ESI-MS) of outer membrane proteins was performed on a Thermo LTQ-Orbitrap-XL mass spectrometer at the QB3/Chemistry Mass Spectrometry Facility at UC Berkeley. Samples were prepared by excising the band of interest from SDS-PAGE gels, followed by in-gel tryptic digestion according to the facility protocol. Data analysis was performed with Thermo Proteome Discoverer (version 1.3) software.

Efflux pump analysis. We used 100um concentrations of the efflux pump inhibitor, Phe-Arg β-naphthylamide dihydrochloride (PaβN), in conjunction with imipenem broth microdilution to assess efflux activity. Both unexposed parental type and 8-hour imipenem exposed samples were tested in triplicate against three concentrations of the inhibitor. $MgS0_4$ was used in a separate set of experiments to ensure that membrane permeability was not contributing to MIC differences. Experiments were repeated twice. Efflux activity was considered significant if there was a 2-fold or greater difference in the imipenem MIC in the presence of the inhibitor (22).

Statistical analysis. Categorical variables were compared by a chi-square or Fisher exact test (2-tailed). Differences in means or proportions were compared with Student's t-test. Differences were considered statistically significant at $p \leq 0.05$.

RESULTS

Pronounced inoculum effect in heteroresistant strains. Imipenem MICs of the eight heteroresistant KPC-producing *K. pneumoniae* strains were in the range of 1-2ug/ml. They increased to 16ug/ml with the 10^6 cfu/ml inoculum, and were greater than or equal to 64ug/ml (the maximum of this test) with the 10^7cfu/ml inoculum--a 32-fold increase (Table 1). These strains all produced colonies within the zone of inhibition of the imipenem ETEST®. Two strains (*K. pneumoniae* BR1, BR20) with high level imipenem resistance at the reference standard (16ug/ml) showed a 4-fold MIC increase with the higher inocula. ETEST® results for these strains showed dense growth of colonies throughout the zone of inhibition. Inoculum effect was not observed among non-KPC-producing *K. pneumoniae* control strains (SF701, SF705, SF519, SF681).

A minor subpopulation of survivors mediates population recovery after lethal imipenem exposure. Bactericidal levels were achieved for all study strains in the first 2 hours of exposure. The mean number of colonies enumerated 2 hours after exposure was $1.6x10^3$ cfu/ml (95% confidence interval (CI), $1.2x10^3$-$2.1x10^3$) for higher inocula samples, and $1.7x10^3$ cfu/ml (95% CI, $7.4x10^2$-$2.6x10^3$) for standard inocula samples ($p>0.05$). The mean number of colonies for non-KPC-producing strains 2 hours after exposure was $1.9x10^4$ cfu/ml (95% CI, $1.1x10^4$-$2.7x10^4$).

For KPC-producing strains, 32 (76%) of 42 time kill samples at a starting inoculum greater than 3.3×10^6 cfu/ml yielded more than 10^9 cfu/ml by 20 hours post-exposure (recovery), while only 4 (11%) of 36 samples below this starting inoculum recovered ($p<0.0001$). None (n=30) of the non-KPC-producing strain samples recovered even at concentrations of imipenem at the MIC.

The number of colonies enumerated 8 hours after exposure ranged from 10^2 to 10^4 cfu/ml, with more survivors enumerated in higher versus standard inocula experiments ($p=0.005$). No significant difference was found in the number of survivors after 8 hours of imipenem exposure between non-KPC-producing strains with higher inocula and KPC-producing strains with standard inocula ($p>0.05$), However, 11% of the latter and none of the former group recovered.

At 2 hours after imipenem exposure under all inocula tested, the survivors were as imipenem-susceptible as their parental strain and did not produce any colonies on imipenem agar plates. At 8 hours of exposure, survivors had several-fold higher imipenem MICs among groups that exhibited recovery at 20 hours, while survivors that showed no recovery had MICs not different from the parental strain (Table 3). Population profiles revealed that an even smaller proportion of the initial 2-hour imipenem exposure survivors recovered (Table 4). For the heteroresistant *K. pneumoniae* strains (BR6, BR7, BR21, BR23, BR28), the frequencies of colonies that grew on imipenem agar in concentrations 8-fold higher (16ug/ml) than the reference standard MIC were

similar for both the 10^7 and 10^6 cfu inocula samples, with a range of $2x10^{-7}$ to $3x10^{-6}$, relative to samples grown on drug-free agar. The frequency of colonies that grew on concentrations of 32ug/ml was $2x10^{-7}$, and 0 to $3x10^{-7}$ for the 10^7 and 10^6 cfu inocula samples, respectively. Non-KPC producing strains grew on imipenem agar at a maximum of 4-fold above the MIC at a frequency of $7x10^{-6}$ to $4x10^{-6}$ of the original inoculum.

KPC enzyme from lysed cells during imipenem exposure does not contribute to population survival. The imipenem MIC for *E. coli* ATCC25922 was 0.125–0.25ug/ml in all of the KPC-producing *K. pneumoniae* culture filtrates from all incubation-time samples, with the exception of supernatant removed from samples 20 hours after exposure to imipenem in a population that recovered; these six samples grew in wells with imipenem concentrations of 8ug/ml, which was the maximum concentration of the test (data not shown). Spontaneous degradation of imipenem was not observed in the test samples until 24 hours of incubation (data not shown).

Increased expression of the *bla*$_{KPC}$ gene does not contribute to high-level resistance in heteroresistant strains. All KPC-producing study strains contained *bla*$_{KPC-2}$. The transcription start site region was 100% identical at the nucleotide level among all strains. The sequence upstream of *bla*$_{KPC}$ in all other strains was 100% identical to the region mapped by Naas et al. to contain three transcription start sites (19).

When *bla*$_{KPC}$ expression was normalized to unexposed samples, change in expression for heteroresistant *K. pneumoniae* strains (BR6, BR7, BR14, BR21) ranged between 0.5-fold to 0.7-fold lower for 2 hour and 8 hour imipenem exposed samples. The four strains had similar expression levels, with the highest expression 2.4-fold higher than the lowest expression (data not shown).

Efflux pump activity does not contribute to survival in lethal doses of imipenem. Imipenem broth microdilution with the efflux pump inhibitor, PaβN, showed no imipenem MIC reduction in any of the unexposed or 8 hour exposed heteroresistant *K. pneumoniae* samples (data not shown).

Porin expression changes contribute to high-level imipenem resistance. The non-KPC-producing *K. pneumoniae* control strain SF519, but none of the KPC-producing *K. pneumoniae* strains, expressed OmpK35. By SDS-PAGE, all heteroresistant *K. pneumoniae* strains exposed to imipenem for 2 hours, as well as their unexposed parental types, showed two bands, which were confirmed by ESI-MS as OmpA and OmpK36 (Table 3, Figure 1). As early as 4 hours post-exposure OmpK36 porin disappeared in some strains. In all 8-hour exposure samples that subsequently recovered, OmpK36 was absent. The imipenem MICs for all such samples was greater than 32ug/ml. The OmpK36 band was present, however, in 8-hour exposure samples of cultures that did not recover, as well as in the non-KPC-producing *K. pneumoniae* control strains. The OmpK36 band was also present in all samples at 2 and 8 hours of drug-free growth. By PCR, in most cases, *ompK35* PCR product was not obtained and evidence of insertions was seen in the *ompK35* coding region in the sequenced PCR products. PCR results for the *ompK36* gene agreed with SDS-PAGE and ESI-MS results. That is, unexposed and 2-hour exposure samples with the OmpK36 protein band yielded *ompK36* PCR product with 100% nucleotide identity to NCBI reference sequence JX310551 (Table 3). For the 4- and 8-hour exposure

samples of one heteroresistant *K. pneumoniae* strain (BR14), the *ompK36* sequence had mutations predicted to encode premature stop codons. In 8-hour exposure samples of several heteroresistant *K. pneumoniae* strains (BR14, BR21, BR28), no *ompK36* product was obtained by PCR, but *ompK37* PCR product with 100% nucleotide identity to NCBI reference sequence KC534871 was obtained. Wild type *ompK36* sequences were obtained for 8-hour exposure samples of several heteroresistant *K. pneumoniae* strains (BR6, BR7, BR19, BR23), even though the OmpK36 protein band was absent. We did not analyze the region upstream of the open reading frame of the porin genes, so we cannot rule out mutations in the promoter or ribosome binding sites which has been noted by others (20, 23).

Imipenem resistant *K. pneumoniae* strain BR20 demonstrated a 2- to 4-fold increase in imipenem resistance when tested at higher inocula. In contrast to the porin profiles of the heteroresistant *K. pneumoniae* strains, a 2-codon nucleotide insertion was found in all imipenem exposed and unexposed samples, and OmpK36 was present by SDS-PAGE analysis in both exposed and unexposed samples. The insertion of GACGGC at position 403 of NCBI reference sequence HM769261 generates Asp135, Gly136 insertions in the L3 channel eyelet loop region described by others (24, 25). Mutations in this region have been predicted to reduce the uptake of carbapenems and cephalosporins, thus increasing the MICs against these drugs through selective restriction without abolishing expression of the porin (26).

In contrast, SDS-PAGE analysis of the constitutively highly resistant *K. pneumoniae* strain BR3 (with no observed inoculum effect) showed no OmpK36 protein even in the unexposed samples.

Stability of high-level resistance is associated with OmpK36 porin loss. Heteroresistant strains were passaged daily on drug-free media and then retested for their imipenem MICs (Table 3). After 8 hours of imipenem exposure, six of the eight strains (BR6, BR14, BR19, BR21, BR26, BR28) showed no decrease in the MIC and had ETESTs® with no zone of inhibition. Two strains (BR7, BR23) reverted to the heteroresistant phenotype, showing colonies in the zone of inhibition of the imipenem ETEST®. Imipenem broth microdilution MIC results showed reversion to the pronounced inoculum effect of the original unexposed strain. SDS-PAGE analysis showed that the strains with no reversion were still missing OmpK36 while the strains that reverted to heteroresistance regained presence of OmpK36. By PCR, the non-reverter strains did not yield *ompK36* amplification products, while the reverter-type strain yielded a sequence with 100% identity to that of the wild type coding region.

KPC enzyme activity is necessary for expression of imipenem heteroresistance. In 100um concentrations of PBA (an inhibitor of KPC hydrolysis), growth in imipenem of heteroresistant KPC-producing *K. pneumoniae* strains expressing *ompK36* was reduced 2-and 4-fold for standard and higher inocula, respectively (Table 3). The highly resistant *K. pneumoniae* strain BR20 (with the OmpK36 channel mutation) grew only in a maximum of 1-2ug/ml imipenem in the presence of PBA, regardless of inocula. In the presence of PBA, imipenem MICs of OmpK36-deficient, the non-reverters BR14, BR19, BR21, and BR28, decreased only 2- to 4-fold, while MICs of non-reverters BR6 and BR26 decreased 4- to 8-fold. However, OmpK36-deficient reverter strains BR7 and BR23 became as susceptible to imipenem as their OmpK36-expressing counterparts (a 16- to 32-fold decrease relative to the test maximum of 32ug/ml

imipenem). OmpK36 was expressed by individual colonies of strain BR7 after dual exposure to PBA and imipenem (at the highest concentration it grew, 2ug/ml), but the porin was still absent in comparison samples exposed to the same dose of imipenem without PBA (Figure 1).

Porin loss does not confer increased resistance to non-β-lactam drugs. Five heteroresistant *K. pneumoniae* strains (BR7, BR14, BR21, BR23, BR28) were tested for resistance to other β-lactam drugs and to unrelated classes of antimicrobial agents to assess the potential contribution of efflux pumps or AmpC-type mechanisms to imipenem resistance (data not shown). Pronounced inoculum effect was observed with cefotaxime (8-fold difference), but not with ceftazidime, two extended spectrum β-lactam drugs. The 2-hour imipenem-exposed samples showed the same MIC for all other drugs tested as their non-exposed counterparts, while the 8 hour imipenem-exposed samples showed a 4-fold increase against cefotaxime and a 2-fold increase against aztreonam. No increased MICs were observed for 8-hour imipenem exposed samples against levofloxacin, gentamicin or trimethoprim-sulfamethoxazole.

Recovered populations comprise subpopulations with heterogeneous imipenem resistance. We analyzed five heteroresistant *K. pneumoniae* strains (BR6, BR14, BR21, BR23, BR28) by serial dilution and plating them after 20 hours of incubation with imipenem, as well as by direct imipenem MIC testing (Table 5). Six to twelve individual colonies/strain were selected for imipenem MIC analysis. Interestingly, most aliquots of these imipenem-exposed total cultures had imipenem MICs in the highly resistant range (>32ug/ml), while the isolated colonies had mixed results with similar MICs and OmpK36 porin profiles as the unexposed parental strains.

DISCUSSION

There is no unified definition for heteroresistance. It is most commonly defined as a characteristic of a bacterial strain population susceptible to a drug according to clinical standards, but that contains subpopulations of much higher resistance. It commonly involves non-heritable phenotypic variability in a genetically homogeneous population (27-30). Heteroresistance was first reported in *Staphylococcus aureus* (methicillin, vancomycin) (31, 32), followed by reports in *Acinetobacter baumanii* (carbapenems, colistin, cephalosporins, penicillins) (33-35), *Pseudomonas aeruginosa* (carbapenems) (36, 37), *Streptococcus pneumoniae* (penicillin) (30), and *Klebsiella pneumoniae* (carbapenems, colistin, chlorhexidine) (38-40). For most, the mechanisms mediating heteroresistance remain elusive or suggest multiple pathways (33, 41-45).

In this study we analyzed phenotypic heteroresistance of KPC-producing *K. pneumoniae* strains to a carbapenem, imipenem. We showed that heteroresistant KPC-producing *K. pneumoniae* strains survive bactericidal concentrations of imipenem from 8- to 32-fold higher than their reference standard MICs. This survival was associated with 1) an inoculum density of at least $3x10^6$ cfu/ml, 2) carriage of the bla_{KPC} gene, and 3) the imipenem-induced generation of a subpopulation of cells with decreased expression of the major outer membrane porin, OmpK36. The survival was not related to other factors such as imipenem degradation or hydrolysis of the drug, or increased expression of bla_{KPC}.

OmpK36 porin loss by KPC-producing strains greatly increased the imipenem MIC. Landman et al. found by real-time RT-PCR analysis that even for *K. pneumoniae* strains with relatively

low expression of *bla*$_{KPC}$, decreased expression of *ompK36* results in substantially higher imipenem MICs (17). Similar quantitative *ompK36* expression studies should be performed with our heteroresistant *K. pneumoniae* strains. Tsai et al. also showed that loss of OmpK36 on its own increased imipenem MICs (46, 47). One would expect OmpK36 loss to be detrimental for bacterial nutrient uptake, but this sacrifice of a subpopulation may have a beneficial outcome for the population as a whole in its defense against antimicrobial stress.

Porin loss in Enterobacteriaceae organisms is commonly reported in clinical treatment cases, and has been shown to occur during the course of carbapenem treatment (48-52). Carbapenem resistance can develop in strains with OmpK36 loss in absence of a carbapenemase (17, 20, 46, 53). Such strains usually express plasmid-mediated AmpC type β-lactamases, or extended spectrum β-lactamases (ESBL) such as CTX-M types. Our KPC-producing *K. pneumoniae* strains nearly all co-harbored *bla*$_{CTX-M-1}$ or *bla*$_{CTX-M-9}$ type ESBLs, and many co-harbored *bla*$_{TEM-1}$ and *bla*$_{SHV-11}$ type β-lactamases. While it is possible that these enzymes contribute to the heteroresistant phenotype, our findings indicate that coordination of *bla*$_{KPC}$ and OmpK36 expression are key components of this phenotype. PBA-mediated inhibition of KPC enzyme activity prevented loss of OmpK36 and population recovery. Moreover, none of the four control strains in this study lacking *bla*$_{KPC}$ (one harbored *bla*$_{CTX-M-15}$) achieved such abrupt imipenem MIC increases with such minor changes in inoculum, and no OmpK36 porin loss was observed under any of the experimental conditions.

There is evidence that carbapenem monotherapy for infections caused by strains with low level resistance leads to high rates of clinical treatment failure (8, 11, 12, 15, 54, 55). There is debate over whether heteroresistant strains are associated with treatment failure (35, 56-60). Nevertheless, our experimental data suggest that the use of carbapenem monotherapy for heteroresistant strains, especially at infection sites where bacterial density may be high and drug penetration suboptimal, may unintentionally lead to induction of higher-level resistance and treatment failure.

The limitation of our study in extrapolating to clinical relevance is that our study is based on in vitro data and with a limited number of strains. However, it does provide some clue into the physiology and importance of resistant subpopulations generated by strains with apparent carbapenem susceptibility upon exposure to bactericidal doses of imipenem. Development of new therapeutic targets, such as those regulating porin expression, for carbapenemase-producing strains is urgently needed, especially for heteroresistant strains, which most likely contribute to the urgent threat of CRE infections.

REFERENCES

1. **Adams-Sapper S, Nolen S, Donzelli GF, Lal M, Chen K, Justo da Silva LH, Moreira BM, Riley LW.** 2015. Rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing Klebsiella pneumoniae. Antimicrob Agents Chemother doi:10.1128/AAC.05100-14.
2. **Anonymous.** Antimicrobial Resistance Global Report on Surveillance, 2014. World Health Organization.
3. **Anonymous.** Antibiotic Resistance Threats in the United States, 2013. US Departent of Health and Human Services, Centers for Disease Control and Prevention,
4. **Bratu S, Tolaney P, Karumudi U, Quale J, Mooty M, Nichani S, Landman D.** 2005. Carbapenemase-producing Klebsiella pneumoniae in Brooklyn, NY: molecular epidemiology and in vitro activity of polymyxin B and other agents. J Antimicrob Chemother **56:**128-132.
5. **Woodford N, Tierno PM, Jr., Young K, Tysall L, Palepou MF, Ward E, Painter RE, Suber DF, Shungu D, Silver LL, Inglima K, Kornblum J, Livermore DM.** 2004. Outbreak of Klebsiella pneumoniae producing a new carbapenem-hydrolyzing class A beta-lactamase, KPC-3, in a New York Medical Center. Antimicrob Agents Chemother **48:**4793-4799.
6. **Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, Alberti S, Bush K, Tenover FC.** 2001. Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae. Antimicrob Agents Chemother **45:**1151-1161.
7. **Bradford PA, Bratu S, Urban C, Visalli M, Mariano N, Landman D, Rahal JJ, Brooks S, Cebular S, Quale J.** 2004. Emergence of carbapenem-resistant Klebsiella species possessing the class A carbapenem-hydrolyzing KPC-2 and inhibitor-resistant TEM-30 beta-lactamases in New York City. Clin Infect Dis **39:**55-60.
8. **Qureshi ZA, Paterson DL, Potoski BA, Kilayko MC, Sandovsky G, Sordillo E, Polsky B, Adams-Haduch JM, Doi Y.** 2012. Treatment outcome of bacteremia due to KPC-producing Klebsiella pneumoniae: superiority of combination antimicrobial regimens. Antimicrob Agents Chemother **56:**2108-2113.
9. **Zarkotou O, Pournaras S, Tselioti P, Dragoumanos V, Pitiriga V, Ranellou K, Prekates A, Themeli-Digalaki K, Tsakris A.** 2011. Predictors of mortality in patients with bloodstream infections caused by KPC-producing Klebsiella pneumoniae and impact of appropriate antimicrobial treatment. Clin Microbiol Infect **17:**1798-1803.
10. **Bratu S, Landman D, Haag R, Recco R, Eramo A, Alam M, Quale J.** 2005. Rapid spread of carbapenem-resistant Klebsiella pneumoniae in New York City: a new threat to our antibiotic armamentarium. Arch Intern Med **165:**1430-1435.
11. **Weisenberg SA, Morgan DJ, Espinal-Witter R, Larone DH.** 2009. Clinical outcomes of patients with Klebsiella pneumoniae carbapenemase-producing K. pneumoniae after treatment with imipenem or meropenem. Diagn Microbiol Infect Dis **64:**233-235.
12. **Arnold RS, Thom KA, Sharma S, Phillips M, Kristie Johnson J, Morgan DJ.** 2011. Emergence of Klebsiella pneumoniae carbapenemase-producing bacteria. South Med J **104:**40-45.
13. **Smith Moland E, Hanson ND, Herrera VL, Black JA, Lockhart TJ, Hossain A, Johnson JA, Goering RV, Thomson KS.** 2003. Plasmid-mediated, carbapenem-

hydrolysing beta-lactamase, KPC-2, in Klebsiella pneumoniae isolates. J Antimicrob Chemother **51:**711-714.

14. **Tenover FC, Kalsi RK, Williams PP, Carey RB, Stocker S, Lonsway D, Rasheed JK, Biddle JW, McGowan JE, Jr., Hanna B.** 2006. Carbapenem resistance in Klebsiella pneumoniae not detected by automated susceptibility testing. Emerg Infect Dis **12:**1209-1213.
15. **Hirsch EB, Tam VH.** 2010. Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. J Antimicrob Chemother **65:**1119-1125.
16. **Nordmann P, Cuzon G, Naas T.** 2009. The real threat of Klebsiella pneumoniae carbapenemase-producing bacteria. Lancet Infect Dis **9:**228-236.
17. **Landman D, Bratu S, Quale J.** 2009. Contribution of OmpK36 to carbapenem susceptibility in KPC-producing Klebsiella pneumoniae. J Med Microbiol **58:**1303-1308.
18. **Wootton M, Howe RA, Hillman R, Walsh TR, Bennett PM, MacGowan AP.** 2001. A modified population analysis profile (PAP) method to detect hetero-resistance to vancomycin in Staphylococcus aureus in a UK hospital. J Antimicrob Chemother **47:**399-403.
19. **Naas T, Cuzon G, Truong HV, Nordmann P.** 2012. Role of ISKpn7 and deletions in blaKPC gene expression. Antimicrob Agents Chemother **56:**4753-4759.
20. **Doumith M, Ellington MJ, Livermore DM, Woodford N.** 2009. Molecular mechanisms disrupting porin expression in ertapenem-resistant Klebsiella and Enterobacter spp. clinical isolates from the UK. J Antimicrob Chemother **63:**659-667.
21. **Carlone GM, Thomas ML, Rumschlag HS, Sottnek FO.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from Haemophilus species. J Clin Microbiol **24:**330-332.
22. **Lamers RP, Cavallari JF, Burrows LL.** 2013. The efflux inhibitor phenylalanine-arginine beta-naphthylamide (PAbetaN) permeabilizes the outer membrane of gram-negative bacteria. PLoS One **8:**e60666.
23. **Cai JC, Zhou HW, Zhang R, Chen GX.** 2008. Emergence of Serratia marcescens, Klebsiella pneumoniae, and Escherichia coli Isolates possessing the plasmid-mediated carbapenem-hydrolyzing beta-lactamase KPC-2 in intensive care units of a Chinese hospital. Antimicrob Agents Chemother **52:**2014-2018.
24. **Garcia-Fernandez A, Miriagou V, Papagiannitsis CC, Giordano A, Venditti M, Mancini C, Carattoli A.** 2010. An ertapenem-resistant extended-spectrum-beta-lactamase-producing Klebsiella pneumoniae clone carries a novel OmpK36 porin variant. Antimicrob Agents Chemother **54:**4178-4184.
25. **Garcia-Fernandez A, Villa L, Carta C, Venditti C, Giordano A, Venditti M, Mancini C, Carattoli A.** 2012. Klebsiella pneumoniae ST258 producing KPC-3 identified in italy carries novel plasmids and OmpK36/OmpK35 porin variants. Antimicrob Agents Chemother **56:**2143-2145.
26. **Alberti S, Rodriquez-Quinones F, Schirmer T, Rummel G, Tomas JM, Rosenbusch JP, Benedi VJ.** 1995. A porin from Klebsiella pneumoniae: sequence homology, three-dimensional model, and complement binding. Infect Immun **63:**903-910.
27. **Rinder H.** 2001. Hetero-resistance: an under-recognised confounder in diagnosis and therapy? J Med Microbiol **50:**1018-1020.

28. **Tomasz A, Nachman S, Leaf H.** 1991. Stable classes of phenotypic expression in methicillin-resistant clinical isolates of staphylococci. Antimicrob Agents Chemother **35:**124-129.
29. **Pfeltz RF, Schmidt JL, Wilkinson BJ.** 2001. A microdilution plating method for population analysis of antibiotic-resistant staphylococci. Microb Drug Resist **7:**289-295.
30. **Morand B, Muhlemann K.** 2007. Heteroresistance to penicillin in Streptococcus pneumoniae. Proc Natl Acad Sci U S A **104:**14098-14103.
31. **Harigaya Y, Ngo D, Lesse AJ, Huang V, Tsuji BT.** 2011. Characterization of heterogeneous vancomycin-intermediate resistance, MIC and accessory gene regulator (agr) dysfunction among clinical bloodstream isolates of staphyloccocus aureus. BMC Infect Dis **11:**287.
32. **Liu C, Chambers HF.** 2003. Staphylococcus aureus with heterogeneous resistance to vancomycin: epidemiology, clinical significance, and critical assessment of diagnostic methods. Antimicrob Agents Chemother **47:**3040-3045.
33. **Cai Y, Chai D, Wang R, Liang B, Bai N.** 2012. Colistin resistance of Acinetobacter baumannii: clinical reports, mechanisms and antimicrobial strategies. J Antimicrob Chemother **67:**1607-1615.
34. **Hung KH, Wang MC, Huang AH, Yan JJ, Wu JJ.** 2012. Heteroresistance to cephalosporins and penicillins in Acinetobacter baumannii. J Clin Microbiol **50:**721-726.
35. **Lee HY, Chen CL, Wang SB, Su LH, Chen SH, Liu SY, Wu TL, Lin TY, Chiu CH.** 2011. Imipenem heteroresistance induced by imipenem in multidrug-resistant Acinetobacter baumannii: mechanism and clinical implications. Int J Antimicrob Agents **37:**302-308.
36. **Oikonomou O, Panopoulou M, Ikonomidis A.** 2011. Investigation of carbapenem heteroresistance among different sequence types of Pseudomonas aeruginosa clinical isolates reveals further diversity. J Med Microbiol **60:**1556-1558.
37. **Pournaras S, Ikonomidis A, Markogiannakis A, Spanakis N, Maniatis AN, Tsakris A.** 2007. Characterization of clinical isolates of Pseudomonas aeruginosa heterogeneously resistant to carbapenems. J Med Microbiol **56:**66-70.
38. **Meletis G, Tzampaz E, Sianou E, Tzavaras I, Sofianou D.** 2011. Colistin heteroresistance in carbapenemase-producing Klebsiella pneumoniae. J Antimicrob Chemother **66:**946-947.
39. **Naparstek L, Carmeli Y, Chmelnitsky I, Banin E, Navon-Venezia S.** 2012. Reduced susceptibility to chlorhexidine among extremely-drug-resistant strains of Klebsiella pneumoniae. J Hosp Infect **81:**15-19.
40. **Pournaras S, Kristo I, Vrioni G, Ikonomidis A, Poulou A, Petropoulou D, Tsakris A.** 2010. Characteristics of meropenem heteroresistance in Klebsiella pneumoniae carbapenemase (KPC)-producing clinical isolates of K. pneumoniae. J Clin Microbiol **48:**2601-2604.
41. **Rohrer S, Maki H, Berger-Bachi B.** 2003. What makes resistance to methicillin heterogeneous? J Med Microbiol **52:**605-607.
42. **Deresinski S.** 2013. The multiple paths to heteroresistance and intermediate resistance to vancomycin in Staphylococcus aureus. J Infect Dis **208:**7-9.
43. **Kohanski MA, DePristo MA, Collins JJ.** 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell **37:**311-320.

44. **Maor Y, Lago L, Zlotkin A, Nitzan Y, Belausov N, Ben-David D, Keller N, Rahav G.** 2009. Molecular features of heterogeneous vancomycin-intermediate Staphylococcus aureus strains isolated from bacteremic patients. BMC Microbiol **9:**189.
45. **Mwangi MM, Kim C, Chung M, Tsai J, Vijayadamodar G, Benitez M, Jarvie TP, Du L, Tomasz A.** 2013. Whole-genome sequencing reveals a link between beta-lactam resistance and synthetases of the alarmone (p)ppGpp in Staphylococcus aureus. Microb Drug Resist **19:**153-159.
46. **Tsai YK, Fung CP, Lin JC, Chen JH, Chang FY, Chen TL, Siu LK.** 2011. Klebsiella pneumoniae outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. Antimicrob Agents Chemother **55:**1485-1493.
47. **Tsai YK, Liou CH, Fung CP, Lin JC, Siu LK.** 2013. Single or in combination antimicrobial resistance mechanisms of Klebsiella pneumoniae contribute to varied susceptibility to different carbapenems. PLoS One **8:**e79640.
48. **Elliott E, Brink AJ, van Greune J, Els Z, Woodford N, Turton J, Warner M, Livermore DM.** 2006. In vivo development of ertapenem resistance in a patient with pneumonia caused by Klebsiella pneumoniae with an extended-spectrum beta-lactamase. Clin Infect Dis **42:**e95-98.
49. **Mena A, Plasencia V, Garcia L, Hidalgo O, Ayestaran JI, Alberti S, Borrell N, Perez JL, Oliver A.** 2006. Characterization of a large outbreak by CTX-M-1-producing Klebsiella pneumoniae and mechanisms leading to in vivo carbapenem resistance development. J Clin Microbiol **44:**2831-2837.
50. **Poirel L, Heritier C, Spicq C, Nordmann P.** 2004. In vivo acquisition of high-level resistance to imipenem in Escherichia coli. J Clin Microbiol **42:**3831-3833.
51. **Song W, Suh B, Choi JY, Jeong SH, Jeon EH, Lee YK, Hong SG, Lee K.** 2009. In vivo selection of carbapenem-resistant Klebsiella pneumoniae by OmpK36 loss during meropenem treatment. Diagn Microbiol Infect Dis **65:**447-449.
52. **Thiolas A, Bollet C, La Scola B, Raoult D, Pages JM.** 2005. Successive emergence of Enterobacter aerogenes strains resistant to imipenem and colistin in a patient. Antimicrob Agents Chemother **49:**1354-1358.
53. **Martinez-Martinez L, Pascual A, Hernandez-Alles S, Alvarez-Diaz D, Suarez AI, Tran J, Benedi VJ, Jacoby GA.** 1999. Roles of beta-lactamases and porins in activities of carbapenems and cephalosporins against Klebsiella pneumoniae. Antimicrob Agents Chemother **43:**1669-1673.
54. **Paterson DL, Ko WC, Von Gottberg A, Mohapatra S, Casellas JM, Goossens H, Mulazimoglu L, Trenholme G, Klugman KP, Bonomo RA, Rice LB, Wagener MM, McCormack JG, Yu VL.** 2004. Antibiotic therapy for Klebsiella pneumoniae bacteremia: implications of production of extended-spectrum beta-lactamases. Clin Infect Dis **39:**31-37.
55. **Lee GC, Burgess DS.** 2012. Treatment of Klebsiella pneumoniae carbapenemase (KPC) infections: a review of published case series and case reports. Ann Clin Microbiol Antimicrob **11:**32.
56. **Deresinski S.** 2009. Vancomycin heteroresistance and methicillin-resistant Staphylococcus aureus. J Infect Dis **199:**605-609.
57. **Falagas ME, Makris GC, Dimopoulos G, Matthaiou DK.** 2008. Heteroresistance: a concern of increasing clinical significance? Clin Microbiol Infect **14:**101-104.

58. **Ikonomidis A, Neou E, Gogou V, Vrioni G, Tsakris A, Pournaras S.** 2009. Heteroresistance to meropenem in carbapenem-susceptible Acinetobacter baumannii. J Clin Microbiol **47:**4055-4059.
59. **Moore MR, Perdreau-Remington F, Chambers HF.** 2003. Vancomycin treatment failure associated with heterogeneous vancomycin-intermediate Staphylococcus aureus in a patient with endocarditis and in the rabbit model of endocarditis. Antimicrob Agents Chemother **47:**1262-1266.
60. **Satola SW, Farley MM, Anderson KF, Patel JB.** 2011. Comparison of detection methods for heteroresistant vancomycin-intermediate Staphylococcus aureus, with the population analysis profile method as the reference method. J Clin Microbiol **49:**177-183.

Table 1**.** *K. pneumoniae* strains used in this study.

			IPM MIC (ug/ml)				
Strain[1]	ST	B-lactamase genes	$5x10^5$ (ref)[2]	$5x10^6$	$5x10^7$	$5x10^8$	ETEST® zone[3,4]
BR6 (HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M}$ (NT)	1-2	16	64	>64	1+
BR7 (HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$	1-2	16	64	>64	1+
BR14(HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M-9}$, *bla*$_{TEM-1}$, *bla*$_{SHV-11}$	2	16	64	>64	1+
BR19 (HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$, *bla*$_{SHV-11}$	2	16	64	>64	1+
BR21 (HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M-1}$, *bla*$_{SHV-11}$, *bla*$_{OXA-1}$	1-2	16	64	>64	1+
BR23 (HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M-1}$, *bla*$_{TEM-1}$, *bla*$_{SHV-11}$	2	16	64	>64	1+
BR26 (HET)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M}$ (NT)	2	16	64	>64	1+
BR28 (HET)	483	*bla*$_{KPC-2}$, , *bla*$_{SHV-11}$	2	16	64	>64	1+
BR1 (HET/RES)	340	*bla*$_{KPC-2}$, *bla*$_{CTX-M}$ (NT)	2-16	16-32	>64	>64	2+
BR20 (RES)	437	*bla*$_{KPC-2}$, *bla*$_{CTX-M}$ (NT)	16	32	>64	>64	2+
BR3 (RES)	340	*bla*$_{KPC-2}$, *bla*$_{SHV-11}$	>64	>64	>64	>64	3+
SF701 (SUSC)	514	none	0.25	0.5	1	4	0
SF705 (SUSC)	1248	none	0.25	0.25	0.5	4	0
SF519 (SUSC)	66	none	0.25	0.25	0.5	4	0
SF681 (SUSC)	392	*bla*$_{CTX-M-15}$	0.25	0.5	1	4	0

[1] Study strain sources: BR, 6 hospitals, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; SF, San Francisco General Hospital, San Francisco, CA, USA; HET: IPM heteroresistant phenotype; RES: high-level IPM resistance; SUSC: IPM susceptible control strains.

[2] CLSI reference standard inoculum.

[3] Number of colonies within zone of inhibition with ETEST®: 0, no colonies; 1+, <50 colonies (within lower region of zone); 2+, >50 colonies (within entire zone); 3+, no zone of inhibition.

[4] Ertapenem ETEST® MIC was similar as for imipenem, with colonies growing within the zone of inhibition.

ST: Multilocus Sequence Type; IPM: imipenem; NT: not typed.

Table 2. PCR primers used in this study.

Primer Target	Primer Name	Sequence 5' – 3'	Expected Amplicon Size (bp)	Reference
bla$_{KPC}$ promoter region	Naas1 Naas11	ACCCTTGCCATCCCGTGTGC AATTGGCGGCGGCGTTATCA	1659	(19)
bla$_{KPC}$	Naas3 Naas2	CTTCAAACAAGGAATATCGTTG ATGCGCCATCGTCAGTGCTCTAC	1040	(19)
ompK36	ompK36-5F ompK36-834R ompK36-782F ompK36-995R	AACTGGTAAACCAGGCCCAG CGTTCAGGCGAACAACACTG AATTTCAGACCTGCGAATGC ACCTGTACGGCAAAATCGAC	829 213	this study
ompK35	ompK35-83 ompK35-1054	AAAACGGCAACAAACTGGAC TGGTAAACGATACCCACGGC	971	this study

Table 3. *Klebsiella pneumoniae* OmpK36 porin analysis and imipenem susceptibility of time kill survivors and unexposed samples.

Strain[1]	IPM (ug/ml)	IPM expos time	IPM MIC STD[2]	IPM MIC HI[2]	IPM MIC PBA[3] (STD, HI)	*ompK36* SNP	OmpK36 status[4]
BR6 (HET)	none	---	1-2	16	0.5, 2	WT	+
	16	2h	1-2	16		WT	+
	16	8h	>32	>32	8,8	WT	-
BR6, passaged	16[5]	8h	>32	>32		WT	-
BR7 (HET)	none	---	1-2	16	1,4	WT	+
	16	2h	1-2	16		WT	+
	16	8h	>32	>32	1,4	WT	+
BR7, passaged	16[5]	8h	2	16		WT	+
BR14 (HET)	none	---	2	16	1,4	WT	+
	16	2h	2	16		WT	+
	16	4h	>32	>32		C430T	-
	16	8h	>32	>32	16,16	C731T, G374A	-
BR14, passaged	16[5]	8h	>32	>32		*ompK37*[6]	-
BR19 (HET)	none	---	4	16	2,2	WT	+
	16	2h	2	16		WT	+
	16	8h	>32	>32	16,16	WT	-
BR19, passaged	16	8h	>32	>32		WT	-
BR21 (HET)	none	---	1-2	16	1,4	WT	+
	16	2h	1-2	16		WT	+
	16	4h	>32	>32		WT	-
	16	8h	>32	>32	16,16	*ompK37*[6]	-
BR21, passaged	16[5]	8h	>32	>32		*ompK37*[6]	-
BR21, std inoculum	none	8h	1-2	8		ND	+
BR21, std inoculum, no recovery	8	8h	2	8		WT	+
BR21, std inoculum, recovery	8	8h	>32	>32		ND	-
BR23 (HET)	none	---	2	16	1,4	WT	+
	16	2h	2	16		WT	+
	16	8h	>32	>32	1,4	WT	-

BR23, passaged	16[5]	8h	2	16		WT	+
BR26 (HET)	None	---	2	16	0.5, 2	WT	+
	16	8h	>32	>32	4,8	WT	-
BR26, passaged	16[5]	8h	>32	>32		ND	ND
BR28 (HET)	none	---	2	16	1,4	WT	+
	16	8h	>32	>32	16,16	WT	-
BR28, passaged	16[5]	8h	>32	>32		*ompK37*[6]	-
BR1 (RES)	none	---	16	>32	4.4	WT	-
	32	8h	>64	>64	16,8	WT	-
BR3 (RES)	none	---	>64	>64	16,>16	WT	-
BR20 (RES)	none	---	16	32	2,4	ins: 403 GACGGC[7]	+
	64	2h	16	32		ins: 403 GACGGC	+
BR20, no recovery	64	8h	16	32		ins: 403 GACGGC	+
BR20, recovery	64	8h	16	32		ins: 403 GACGGC	+
SF701, SF705 (CTL)	none	---	0.5	0.5		WT	+
	2,1,0.5	8h, 24h	0.5	0.5		WT	+

[1]All samples selected from higher inocula time kill experiments unless otherwise noted; passaged: 8-hour IPM exposed samples were passaged daily on drug-free MH plates for at least 7 days. HET: IPM heteroresistant phenotype; RES: high-level IPM resistance; CTL: IPM susceptible control strains.

[2]IPM MIC results (ug/ml) for starting inocula, $5x10^5$ cfu/ml (STD), and $5x10^6$ cfu/ml (HI).

[3]IPM MIC results (ug/ml) for starting inocula $5x10^5$(STD)/$5x10^6$ (HI) cfu/ml in presence of 100um phenylboronic acid (PBA).

[4]SDS-PAGE analysis of bands corresponding to OmpK36 porin.

[5]Original IPM exposure concentration prior to drug-free passage.

[6]*ompK37* gene product (100% identity to NCBI KC534871) obtained by PCR with *ompK36* primers.

[7]100% identity to NCBI HM769261.
SNP: single nucleotide polymorphisms detected by PCR analysis; WT: wild-type sequence (NCBI JX310551); ND: not determined; IPM: imipenem.

Table 4. Frequency of heteroresistant subcolonies for select heteroresistant KPC-producing *K. pneumoniae* strains.

Strain	Highest IPM concentration (ug/ml)	Frequency, 10^7 cfu inocula	Frequency, 10^6 cfu inocula	Fold-increase in IPM MIC[1]
BR6, BR7, BR21, BR23, BR28 [2]	16	$2x10^{-7} – 3x10^{-6}$	$3x10^{-7} – 1x10^{-6}$	8
	32	$2x10^{-7}$	$3x10^{-7}$	16
2h exposure samples, BR6, BR7, BR21[3]	16	$1- 2x10^{-6}$	$3x10^{-7} – 1x10^{-6}$	8
	32	$2x10^{-7} – 1x10^{-6}$	$3x10^{-7}$	16
SF519, SF701 [4]	1, SF519; 2, SF701	$4x10^{-6} – 7x10^{-6}$	$4x10^{-6} – 7x10^{-6}$	4
SF681[5]	1	$2-3x10^{-6}$	$2-3x10^{-6}$	4

[1] CLSI reference standard IPM MIC.

[2] Heteroresistant KPC-producing *K. pneumoniae* strains

[3] Heteroresistant KPC-producing *K. pneumoniae* strains, exposed for 2 hrs to imipenem

[4] Non-KPC-producing *K. pneumoniae* strains

[5] CTX-M-producing *K. pneumoniae* strain

Table 5. Imipenem MICs of individual colonies selected from 20-hour imipenem-exposed cultures reveals presence of heteroresistant subpopulations.

Highest IPM concentration grown (ug/ml)[1]	Individual colonies (n), Strain (reverter type[2])					ompK36 protein[3]
	BR14 (non-rev)	BR21 (non-rev)	BR6 (non-rev)	BR23 (rev)	BR28 (rev)	
2	18	13	17	6	0	+
4	2	1	1	0	0	+
8	1	1	0	0	0	+
16	3	4	0	0	0	-
32	16	18	0	12	24	-
IPM MIC, 20h total culture	>32	1->32	2->32	>32	>32	Variable[4]
as above, >7 days drug-free passage	>32	>32	ND	>32	>32	ND

[1]Tests were performed with CLSI reference standard inocula ($5x10^5$ cfu/ml); IPM: imipenem.

[2] Reverter type is defined as non-reverter (non-rev) if conversion to high-level IPM resistance upon IPM exposure was retained, or reverter (rev) if the original IPM heteroresistant phenotype was observed after one week of daily drug-free passage.

[3] Presence of OmpK36 determined by SDS-PAGE.

[4] OmpK36 was present in all except one of the whole culture samples tested (BR21 strain).

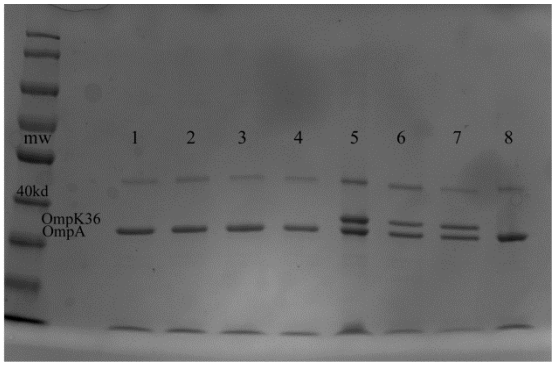

Figure 1. Outer membrane fractions of 8 hour imipenem-exposed OmpK36-deficient heteroresistant *K. pneumoniae strain* BR7 samples analyzed by SDS-PAGE. Identity of OmpA and OmpK36 confirmed by EIS-MS with locations marked on image; 170kDa molecular weight (mw) ladder shown. Lanes 1-4, samples from 2ug/ml imipenem wells; lanes 5-7, samples from 2ug/ml imipenem and 100um KPC enzyme inhibitor, PBA, wells (maximum concentration of sample growth); lane 8, BR7 wild type control, initially expressing OmpK36, exposed to 16ug/ml imipenem. Note that strain BR7 abolished production of OmpK36 with lethal imipenem exposure, but resumes production of OmpK36 upon drug-free passage (refer to text).

CHAPTER TWO. Potential novel regulatory control of outer membrane porins, *ompK35* and *ompK36,* in carbapenem heteroresistant *Klebsiella pneumoniae*.

INTRODUCTION

In our recent study of carbapenem heteroresistance in KPC-producing *Klebsiella pneumoniae* strains, we found that survival in lethal concentrations of imipenem, and conversion to high-level resistance is mediated through the coordination of KPC enzyme hydrolysis, bacterial inoculum, and diminished production of the outer membrane porin, OmpK36 (1). These heteroresistant strains had reduced susceptibility (1-2ug/ml) to imipenem, a carbapenem drug, according to standard laboratory testing. However, upon single exposure to imipenem, we were able to isolate distinct subpopulations with MICs ranging from 1 ug/ml to greater than 64ug/ml.

We previously described two types of *ompK36* regulation among our heteroresistant strains. The majority (6 of 8) of the strains permanently abolished OmpK36 upon lethal imipenem exposure. We found evidence of mobile insertion element interruptions in the coding region of *ompK36* in these strains. Two strains however, reverted to the heteroresistance phenotype and resumed production of OmpK36 once imipenem exposure was removed. We wish to explore the differences in *OmpK36* regulation in these two groups, and to address whether the heteroresistant phenotype is mediated by one or more genes in the chromosome or on a plasmid, or the interaction among them.

We conducted this study by sequencing whole genomes of heteroresistant and highly resistant *K. pneumoniae* KPC-producing strains. We also compared characteristics and genotypes of carbapenem heteroresistant *Escherichia coli* and *Enterobacter cloacae* carrying bla_{KPC} with those of heteroresistant KPC-producing *K. pneumoniae*. We found a high level of genotypic diversity between heteroresistant strains with similar phenotypes. We also found genetic similarities between strains with different imipenem resistance phenotypes. Analysis of the regions immediately adjacent to outer membrane porin genes, *ompK35* and *ompK36*, provided preliminary evidence of potential regulatory mechanisms involved in the expression of heteroresistance. We also found evidence for the role of genes other than bla_{KPC} in the Tn*4401* structure that may mediate the heteroresistance phenotype.

METHODS

Study strains. Five of the imipenem heteroresistant KPC-producing *Klebsiella pneumoniae* strains in this study (BR7, BR14, BR21, BR23, BR28) were characterized in our previous study (1). BR14ΔK36 is an isogenic OmpK36-deficient, imipenem resistant mutant of heteroresistant strain BR14. CBpB3 is a transformant with the plasmid extracted from the BR3 strain. The host strain, CB49, belongs to the same MLST clonal group as BR28 (ST483). BR1 and BR2 are *K. pneumoniae* and *E. coli*, respectively, which have some phenotypic characteristics of imipenem heteroresistance, including presence of colonies within the zone of inhibition of imipenem ETEST® (Table 6). These 2 strains have higher imipenem resistance levels at the reference standard inocula than the defined heteroresistant strains in the study. BR10 is a heteroresistant *E. cloacae* strain. BR3 is a highly imipenem resistant strain of the same MLST clonal group as BR1 (ST340). SF519, a drug-susceptible *K. pneumoniae* bloodstream infection isolate collected

from San Francisco General Hospital, was used as a control strain. All of the other study strains were obtained from rectal swabs, bloodstream and urinary tract infections collected from hospitals in Brazil.

Imipenem susceptibility testing. Antimicrobial susceptibility testing was performed by broth microdilution in accordance with the standards set by Clinical and Laboratory Standards Institute (CLSI) and ETEST® (bioMérieux, Marcy l'Etoile, France). Imipenem (Sigma-Aldrich, St. Louis, MO) was used as the representative carbapenem drug in all experiments. Inoculum-dependent increases in the minimum inhibitory concentration (MIC) for imipenem were determined based on the CLSI reference standard starting inoculum of $5x10^5$ cfu/ml compared to inocula of $5x10^6$, $5x10^7$ and $5x10^8$cfu/ml. An inoculum effect was considered positive if the higher test inocula resulted in an 8-fold or greater increase in the imipenem MIC.

Analysis of outer membrane proteins. Outer membrane proteins were isolated according to the method of Carlone et al (2). Briefly, samples were grown in nutrient broth or MH broth at OD_{600} of 0.6, centrifuged at 5000xg for 10 minutes, washed and resuspended in 10mM HEPES buffer, pH 7.4 and sonicated. The sodium N-lauroyl sarcosinate insoluble outer membrane porins were selectively obtained by incubation in 10mM HEPES buffer with 2% sarkosyl, followed by 30 minute centrifugation at 15,600xg. Samples were boiled and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gels (Bio-Rad, Hercules, CA). Controls included drug-susceptible *Klebsiella pneumoniae* strains.

ESI-MS. Electrospray ionization mass spectrometry (ESI-MS) of outer membrane proteins was performed on a Thermo LTQ-Orbitrap-XL mass spectrometer at the QB3/Chemistry Mass Spectrometry Facility at UC Berkeley. Samples were prepared by excising the band of interest from SDS-PAGE gels, followed by in-gel tryptic digestion according to the facility protocol. Data analysis was performed with Thermo Proteome Discoverer (version 1.3) software.

Whole genome sequencing. Whole genome sequencing was performed at the QB3/Vincent J. Coates Genomics Sequencing Facility at UC Berkeley. Library preparation for sequencing followed a standard protocol for compatible libraries (Wafergen Biosystems, Fremont, CA). Samples were fragmented with a Covaris S220 ultrasonicator to generate an average insert size of 600 bp. The sample fragmentation length was verified with an Agilent Bioanalyzer, then loaded on the Wafergen Apollo 324 system. Wafergen PrepX Library kits were used for end-repair, A-tail addition, adapter ligation, and size selection with AMPure XP beads. Sample concentration was quantified with a Qubit fluorometer. Libraries were PCR amplified to incorporate indexes and flow cell binding regions. Final libraries were quantified by Qubit, Bioanalzyer and qPCR, then sequenced via a 300 base pair, paired-end run on a MiSeq instrument by V3 chemistry and standard Illumina analysis software. Paired reads were assembled into contigs with Geneious version 8.0.4 (Biomatters, Ltd.), which were then submitted for BLAST® search of the National Center for Biotechnology Information (NCBI) database. Contigs were uploaded for annotation to the Rapid Annotation using Subsystem Technology (RAST) server at rast.nmpdr.org (3), and for detection of plasmid (4) and drug resistance (5) genes to the PlasmidFinder and ResFinder servers, respectively, at the Center for Genomic Epidemiology managed by the Technical University of Denmark (DTU).

RESULTS

Inoculum-dependent expression of imipenem resistance. As we previously reported, the heteroresistant *K. pneumoniae* strains (BR7, BR14, BR21, BR23, BR28) showed an inoculum-dependent, linear increase in imipenem resistance ranging from 1 – 2ug/ml for the lowest inocula to >32 ug/ml for the highest (Table 6) (1). Compared to the heteroresistant *K. pneumoniae* strains, *E. cloacae* BR10 showed much less increase in resistance with increased inocula until 10^7 cfu/ml, at which point there was an abrupt increase in the MIC to >32ug/ml. It produced colonies within the zone of inhibition of imipenem ETEST® (Table 6, Figure 2). While *K. pneumoniae* BR1 and BR3 were both imipenem resistant, they differed in their expression of resistance. BR3 was frankly resistant (>32ug/ml) at all inocula tested, while the imipenem MIC of BR1 was highly variable between tests at the CLSI reference standard inocula (2-16 ug/ml), and produced colonies in the zone of inhibition of imipenem ETEST®, but abruptly increased its imipenem MIC with slightly higher inocula to >32ug/ml. *E. coli* BR2 and the CBpB3 transformant were resistant at the reference standard inoculum, and showed linear, inoculum-dependent increases in the MIC, but produced colonies in the zone of inhibition of imipenem ETEST®. In fact, strain BR2 appears to be relatively susceptible according to imipenem ETEST® results (Figure 2). The *K. pneumoniae* host strain CB49 was completely susceptible to imipenem at all inocula.

Limited commonality in the drug resistance genes between the heteroresistant KPC-producing strains. All of the study strains were multidrug resistant, and with the exception of *E. coli* BR2, were found to carry genes encoding resistance to a wide diversity of drug classes (Table 7). Only 5 of the study strains carried $bla_{\text{CTX-M}}$ types. BR7 and BR23 carried $bla_{\text{CTX-M-15}}$, BR14 and BR10 carried $bla_{\text{CTX-M-9}}$, and CB49 and the CBpB3 transformant carried $bla_{\text{CTX-M-8}}$.

Notable differences in the blaKPC-containing Tn4401 genetic region. All strains were found to contain $bla_{\text{KPC-2}}$ (Table 7). The gene was found in all strains in the typical Tn*4401* structure with IS*Kpn7* transposon, including the *istA* and *istB* genes upstream of the bla_{KPC} promoter and coding region, followed by IS*Kpn6* transposon (Figure 3, GenBank accession no. CP004367). With the exception of the CBpB3 transformant, the Tn*440*1 structure was inserted in the *nuc* gene in all of the strains. The insertion occurred between 2 sets of core genes described by Chen et al, providing plasmid replication, regulation, transfer, stability, exclusion of DNA entry, and inhibition of degradation and restriction modification (6).

The more highly imipenem-resistant strains (*K. pneumoniae* BR1, BR3, CBpB3, *E. coli* BR2), and the heteroresistant *E. cloacae* strain BR10, had notable differences in the Tn*4401* structure (Table 8, Figure 3). In BR1 and BR3, a portion of the *tnpA* gene of IS*Kpn6* preceded the left inverted repeat of Tn4401 that usually begins the structure. This was followed *rep*-like, *rom,* and *excl1* genes, followed by the typical Tn*4401* arrangement. The BR2 strain had the *rep*-like gene preceding Tn*4401*as well, but the IS*Kpn6* mediated insertion of *rom* and *excl1* was found following the intact copy of IS*Kpn6* at the end of the Tn*4401* structure.

The *K. pneumoniae* CB49 strain was transformed with the plasmid extracted from *K. pneumoniae* BR3. Genomic analysis showed that the transformant only received the Tn*4401* region, which was inserted into an existing plasmid carried by the host strain. The Tn*4401*

structure of the transformant had the *rep*-like, *rom*, and *excl1* genes from BR3, but was arranged in the same manner as *E. coli* BR2.

The *E. cloacae* BR10 strain had a 35kb insertion of plasmid stability and conjugal transfer genes into the IS*Kpn6* region. No portion of IS*Kpn6* was translocated in this strain and the genetic arrangement of Tn*4401* was otherwise the same as in the *K. pneumoniae* heteroresistant strains.

Limited commonality in the plasmid-borne genes between the heteroresistant KPC-producing strains. The plasmids in all of the strains were highly mosaic, containing regions of high identity to several published sequences in the NCBI database (Table 9). Based on nucleotide identity, the most common plasmid-borne features we found in all of the heteroresistant strains were associated with the *bla*$_{KPC}$-containing Tn*4401* genetic structure. This region was associated with the IncN plasmid in heteroresistant *K. pneumoniae* strains BR14, BR21, BR23, BR28, in *E. coli* strain BR2, and in the resistant *K. pneumoniae* strains BR1 and BR3 (Table 8). Heteroresistant *K. pneumoniae* BR7 contained only the FIB pQIL-type replicon, and in *E. cloacae* BR10, it was associated with IncX3.

The IncN region surrounding the Tn*4401* structure was present in all of the study strains with the exception of the CBpB3 transformant, and most closely matched the 54,605 bp plasmid, pKPC_FCF/3SP (GenBank accession no. CP004367). Heteroresistant strain BR7 was missing the 20 kbp region from the IncN replication protein through a truncated copy of *traI*. *E. cloacae* BR10 only had the genes following Tn*4401* through *traE*. The genes in the common alignment regions encoded core plasmid functions as described above.

Heteroresistant strains BR21, BR23, BR28, and highly resistant strains BR1 and BR3 all shared the same FIB(K) and FII(K) plasmid replicon types, and all seem to be located on the same plasmid with IncN. We are performing conventional PCR analysis to confirm these results. Heteroresistant strain BR14 had different FIB and FII replicon types, likely together on a plasmid, but separate from the IncN plasmid in this strain. *E. coli* BR2 had FIB, FIA, and FII types similar to other *E. coli* strains, which do not seem to be on the same IncN plasmid in this strain.

Analysis of the outer membrane porin *ompK35* genetic region. We previously found by SDS-PAGE analysis that none of the study strains (BR7, BR14, BR21, BR23, BR28, BR1, BR3) produced the major outer membrane porin, OmpK35 (1). Analysis of the genetic region surrounding *ompK35* (homolog of *ompF* in *E. coli*, *omp35* in *E. cloacae*) revealed differences between the study strains, although the basic structure was remarkably similar (Figure 4, Table 8). The coding region of the *ompK35* gene had no mobile insertion element interruptions in any of the strains. In *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR23, and BR28), a cluster of mobile genetic elements was found between the asparaginyl tRNA synthetase gene and the *ompK35* coding region. These genetic elements included IS*Ecp1*, with a disrupted portion of the *pgtP* gene (GenBank accession no. AB914799) and an element with 100% identity to a transposase annotated as *orfB* (GenBank accession no. CP008933).

Upon closer analysis of the putative *ompK35* promoter region in the *K. pneumoniae* study strains (BR7, BR14, BR14ΔK36, BR21, BR23, BR28), *E. cloacae* BR10 and the transformant CBpB3, we found four sites that were highly similar to the phosphorylated OmpR (OmpR~P)

binding sites described by Yoshida et al. (7) (Figure 4). We found sites with 100% identity in *E. coli* BR2. We also discovered additional putative OmpR~P binding sites in all of the heteroresistant strains (BR7, BR14, BR21, BR23,BR28) and in BR14ΔK36, caused by the insertion of the IS*Ecp*1 and *orfB* elements (Table 10). The first set was located in the original position prior to the IS*Ecp1* insertion. Two of the three activating binding sites contained single nucleotide polymorphisms (SNPs). However, all of the nucleotides denoted as critical for high-affinity binding were conserved in this set of binding sites (7). The canonical -35, -10, and +1 transcription start site, but not the Shine-Delgarno sequence, were also preserved in the original region prior to the insertion. The putative site within the IS*Ecp1* element also had SNPs and contained most of the nucleotides considered critical for high-affinity OmpR~P binding. All of the binding sites conserved the G nucleotide in position 11 of the sequence considered to be the most critical. Additionally, a second insertion element, *orfB*, followed the IS*Ecp1* insertion, and created what appears to be a high affinity OmpR~P binding site, and second set of transcription elements close to the *ompK35* coding region.

In strain BR23, the IS*Ecp1* element appears to have mediated a transposition of another partial copy of IS*Ecp1* and bla_{TEM} into the region following the asparaginyl tRNA synthetase gene (Figure 4). The regulatory elements were otherwise intact and identical to the other heteroresistant *K. pneumoniae* strains. In strain BR7 however, IS*Ecp1* seems to have reversed the orientation of the asparaginyl tRNA synthetase gene and the OmpR~P regulatory elements so that they are transcribed in the opposite orientation. In addition, an IS*1* element and a 72kb genetic region were inserted between the gene encoding Lrp and the IS*Ecp1*/*orfB* insertions upstream of *ompK35*. Our laboratory is performing conventional PCR analysis to confirm these arrangements.

K. pneumoniae strains BR1 and BR3 contained an IS*1* element directly upstream from the *ompK35* coding region that ended 23 nucleotides from the translation start site which disrupted all of the transcription recognition sites. *E. cloacae* BR10 had a 314 bp hypothetical protein adjacent to the *omp35* coding region with 99% identity to a portion of a Gram-negative porin (GenBank accession no. CP003737).

Analysis of the outer membrane porin ompK36 genetic region. The genetic arrangement of the *ompK36* region in all of the heteroresistant *K. pneumoniae* study strains (BR7, BR14, BR21, BR23, BR28) was 100% identical (Figure 5). The genes in this region encode a sensor kinase (RcsC), a DNA-binding capsular synthesis response regulator (RcsB), phosphotransferase intermediate (RcsD), OmpK36, and thiamine biosynthesis (ApbE*)*. It also contained the OmpR regulatory binding sites and the *micF* regulatory region. The *E. cloacae* BR10 strain had a region with the same genes (77% identity). The *E. coli* BR2 strain region contained these genes with 99% identity, but contained an additional histidine kinase preceding this region (AtoS) rather than DNA gyrase that preceded it in the other study strains (not shown). While *E. cloacae* BR10 was found to abolish Omp36 production during lethal imipenem exposure, *E. coli* BR2 did not abolish OmpC under these conditions (Table 8).

In the *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR23, BR28), the three OmpR~P binding sites in the *ompK36* region were also highly similar to those described by Yoshida et al (7). We found SNPs in some of these putative binding sites (Table 10). All but 1 of the nucleotides denoted as critical for high affinity binding of OmpR~P were conserved, and

this SNP was found in the lower affinity segment of the site. *E. cloacae* BR10 likewise had sites that contained all of the critical binding nucleotides, and *E. coli* BR2 had sites with 100% identity (not shown).

The OmpK36 mutant, BR14ΔK36, had an IS*Kpn18* insertion element interrupting the middle of the *ompK36* coding region, which explained its absence with SDS-PAGE protein analysis. The structure of the region surrounding *ompK36* was otherwise 100% identical to the heteroresistant *K. pneumoniae* strains.

The resistant strains, BR1 and BR3, also did not produce the OmpK36 protein according to SDS-PAGE analysis (1). The genes found in the region surrounding *ompK36* in both strains were present with 100% identity to the *K. pneumoniae* heteroresistant strains. The *ompK36* coding region was uninterrupted, but an IS*5*-like insertion element was found directly upstream (within 117 nucleotides) of the *ompK36* promoter region and disrupted all of the putative OmpR regulatory elements. Interestingly, the IS*5*-like element was also present on the plasmid contained in these two strains and in the *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR28). In the BR1 strain, this element translocated the RcsCBD genes to another part of the chromosome which eliminated the transcriptional recognition elements upstream of *ompK36*.

The *ompK36* region in the transformant strain CBpB3, and its host strain CB49, contained the same genes with 99% identity with the exception of *ompK36*, which has only 94% identity to the other strains (Table 8). In SDS-PAGE analysis, imipenem-exposed samples of CBpB3 produced the OmpK36 protein, unlike the heteroresistant strains (BR7, BR14, BR21, BR23, BR28), which we previously showed do not express OmpK36 when exposed to lethal doses of imipenem. The *K. pneumoniae* strain BR20, characterized in a previous study, contained a mutation that modified the barrel of the OmpK36 porin, allowing the strain to restrict the inflow of imipenem (1). This may be the case with OmpK36 in CBpB3.

An alternate porin expressed in highly resistant strain, *K. pneumoniae* BR1. An additional 43kDa prominent band was present in SDS-PAGE protein gels from outer membrane extracts of BR1 (not shown). ESI-MS analysis most closely matched the excised protein band to an outer membrane protein in *K. pneumoniae* strain 342 (GenBank accession no. CP000964, protein ID ACI07397). A tblastn search yielded an outer membrane protein with 100% identity on the amino acid level, and 94% identity on the nucleotide level to a gene in BR1 annotated as outer membrane porin N. A BLAST comparison of the nucleotide sequence in BR1 yielded 100% sequence identity with a gene in strain NJST258-1 (GenBank accession no. CP006923) annotated as outer membrane protein S1 precursor. This porin has been characterized and the functional properties were found to be similar to OmpC, OmpK36, and OmpS1 in *Salmonella typhi* (8). The influx of mono- and disaccharides was found to be more similar to the OmpF/OmpK35 porin. Interestingly, when BR1 is exposed to imipenem, the band becomes absent in SDS-PAGE protein gels. The *ompN* gene is present in BR3 with 100% identity, and in BR7, BR14, BR14ΔK36, BR21, BR23, BR28, and CBpB3 with 99% identity. However, the protein band corresponding to the size of this porin has not been seen in the outer membrane extracts of any of these other strains.

IS elements in outer membrane porin regions present in other genetic regions. The IS*Ecp1* element in the *ompK35* region of the *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR23, and BR28) is 100% identical to the element often associated with the promoter region of

several groups of the β-lactamase gene, *bla*$_{CTX-M}$. This element was present in the *ompK35* region in strains BR21 and BR28 that do not carry *bla*$_{CTX-M}$ genes. In strains BR7 and BR23 that carry *bla*$_{CTX-M-15}$, IS*Ecp1* preceded this gene, but was disrupted by an IS*903b* element. In strains BR14 and BR14ΔK36, a full, uninterrupted copy of IS*Ecp1*preceded *bla*$_{CTX-M-9}$. (In CB49 and the transformant CBpB3, *bla*$_{CTX-M-8}$ was preceded by an IS*10* element, and in *E. cloacae* BR10, *bla*$_{CTX-M-9}$ was preceded by an IS*CR1* element.)

The IS*Ecp1*element was also found in strains BR1 and BR3. Neither carry *bla*$_{CTX-M-type}$ genes. The element was found to disrupt the *pgtP* gene in both strains, the same gene whose fragment was found with this element in the *ompK35* region of the *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR23, and BR28). Interestingly, in the latter strains, a complete copy of *pgtP* gene was present on the chromosome, uninterrupted by IS*Ecp1*or any other insertion element. IS*Ecp1* was not found in CBpB3, BR2 or BR10.

An IS*1* element with 100% identity to the element in the *ompK35* region of strains BR1 and BR3 was found in *K. pneumoniae* strains BR7, BR14, BR14ΔK36, BR21, BR23, and BR28. In these strains the element was adjacent to a variety of genes including lytic murein transglycosylase, chloramphenicol and β-lactamase genes, phage proteins, tRNA genes, iron transport, and other mobile genetic elements (not shown). In BR1 and BR3, the element was only found in conjunction with the *ompK35* region. An IS*1*-like element with 99% identity was found in *E. cloacae* BR10 and *E. coli* BR2 strains, adjacent to adhesion and invasion genes.

The IS*Kpn18* element disrupting *ompK36* in BR14ΔK36 was found in other *K. pneumoniae* strains. In BR14ΔK36 and BR14, it was also found to disrupt the *ygpG* gene. In strains BR7, BR23, BR28, BR1, and BR3 it disrupted an FKBP-type peptidyl-prolyl cis-trans isomerase gene. Additionally in BR1 it was found adjacent to the alternately expressed porin gene, *ompN*. This element was not found in the region of the *ompN* gene in any of the other study strains. The IS*Kpn18* element was not found in CBpB3, BR2 or BR10.

DISCUSSION

In this study, KPC-producing *K. pneumoniae* strains from 6 hospitals in Brazil exhibited a highly similar imipenem heteroresistance phenotype. Seven of these strains were clonally related and 1 was from another clonal group. Our previous characterization showed that the imipenem heteroresistance phenotype was mediated through the common elements of carriage of *bla*$_{KPC}$ (we showed that variation in expression did not mediate the phenotype), a slightly elevated inoculum size, a lethal dose of imipenem, and abolished production of a major outer membrane porin, OmpK36 (1). The connection between porin expression and carbapenem resistance has been shown by others (9-16), but our findings elucidated the timing and conditions of OmpK36 production from which we can further explore the underlying mechanisms.

The data provided by whole genome sequencing uncovered more diversity than anticipated among our heteroresistant study strains, even between those of the same MLST clonal group. Further, in strains that contained the same plasmid replicons, the actual genetic content of the plasmids was incredibly mosaic, with regions matching those of several well-characterized plasmids. Between strains, there were differences in insertion elements, transpositions, deletions, and translocations to the chromosome. Our initial goal was to use whole genome sequencing to identify a common set of plasmid-borne genes that might be mediating the

expression of heteroresistance. The region with the highest level of similarity among all of the study strains was that of the *bla*$_{KPC}$-containing Tn*4401*. This structure is a 10kb region with transposons IS*Kpn7*, IS*Kpn6* and transposition helper genes *istA* and *istB*. Different isoforms of the structure have been reported that contain mutations in the multiple promoter region (17). Recently, reports have described the rearrangements of the structure, as we observed in strains BR1, BR2, BR3 and the CBpB3 transformant (6, 18-20). The IS*Kpn6*-mediated rearrangements occurred in all 4 of the more resistant KPC-producing strains. An insertion of core plasmid maintenance and stability genes occurred within IS*Kpn6* in heteroresistant strain BR10 without rearranging the Tn*4401* structure. It is currently unknown whether the IS*Kpn6*-mediated rearrangements of Tn*4401* in BR1, BR2, BR3 and CBpB3 contribute to higher imipenem resistance, and will require further testing. Although the Tn*4401* region seems to be the most significant of the shared plasmid content among all heteroresistant strains, we cannot rule out the commonality of genes with similar functions because our analysis was focused on nucleotide identity. However, we have begun to undertake mutational analysis of the strains which indicates that a cluster of metabolic genes on the chromosome is an important part of this phenotype. Detailed analysis of these results are reported in the chapter that follows.

Our previous findings showed that abolished production of OmpK36 was a critical component of resistance to high concentrations of imipenem (1). Since the plasmid content of the strains did not yield obvious clues explaining differences between heteroresistant and highly resistant strains, and there were no correlations with carriage of specific drug resistance genes, analysis of the regions encoding the outer membrane porins was the obvious next step to explore the regulation of heteroresistance.

The major outer membrane porins produced by *K. pneumoniae* are OmpK35 and OmpK36. These are homologs of OmpF and OmpC, respectively, produced in *E. coli* (7, 21-24). Both allow passive diffusion of hydrophilic nutrients across the outer membrane. As these channels can also allow unintended solutes such as drugs and bile salts to enter the cell, production of these two porins is tightly, and reciprocally regulated. This regulation was first described in *E. coli* (7, 24-29). OmpK35 is the larger of the two channels, and in an environment of toxic compounds, it is the first porin to stop being produced (21). It is preferentially produced in environments of low osmolarity where nutrients are scarce and the larger pore is more advantageous (22, 24). Conversely, OmpK36 is preferentially produced in medium to high osmolarity environments. Clinical strains commonly do not produce OmpK35, which is consistent with our previous findings in regard to the study strains (9, 30). This is likely an adaptive response to the high osmolarity environment in the human intestine (15).

One form of regulation is through *micF*, a 93 nucleotide, untranslated complementary RNA that that exerts post-transcriptional control of *ompK35* (21, 28). *micF* is located upstream of *ompK36* and is transcribed in response to a variety of stressors through the presence of sites in its promoter region that can be bound by transcriptional activators including OmpR, leucine-responsive protein (Lrp), MarA, SoxS, and Rob (21, 28). In high osmolarity conditions, regulation of *ompK35* and *ompK36* expression occurs primarily through the transcriptional control of the EnvZ-OmpR two component system encoded by *ompB* (7, 21, 27, 29). EnvZ is a sensor protein that has both kinase and phosphatase activity. Phosphorylated OmpR (OmpR~P) is a transcription factor that controls the reciprocal expression of *ompK35* and *ompK36* through cooperative binding to sites upstream of the respective promoters (7). In low osmolarity

environments, the amount of OmpR~P is low, so it is bound primarily to high-affinity sites in the *ompK35* promoter region. In higher osmolarity environments, OmpR~P increases, then binds to sites in the *ompK36* region and also to a site in the *ompK35* region that blocks transcription of the gene by forming a loop-OmpR~P complex.

The *ompK35* promoter region contains four OmpR binding sites, while the *ompK36* region contains three. We identified highly similar promoter regions and regulatory binding sites in *K. pneumoniae*, which has been reported by others (15). OmpR binding sites were found in all of the study strains with the exception of sites in the *ompK36* region in BR1 and BR3, which were interrupted by an IS*5*-like insertion element. The regions determined to be critical for high-affinity binding of OmpR~P were highly conserved in all strains (7). In the *ompK35* region of the *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR23, BR28) the insertion of the IS*Ecp*1 and *orfB* elements created three additional putative OmpR~P binding sites. We identified a second set of putative transcription elements within the *orfB* element, just upstream of the *ompK35* coding region. The IS*Ecp1* element is usually reported in conjunction with the $bla_{\text{CTX-M}}$ gene, encoding an extended spectrum β-lactamase. In many cases this element has been found to provide overexpression of $bla_{\text{CTX-M}}$ and increased resistance to extended spectrum cephalosporin drugs (31-33). The IS*Ecp1* element in the *ompK35* region contains the -35 and -10 elements with 100% identity to those described by Poirel et al. (31). Our laboratory plans to conduct experiments to determine the transcriptional function those sites may provide in our strains.

We conducted analysis of these regulatory elements to explain the two distinct types of OmpK36 production observed in the heteroresistant *K. pneumoniae* study strains. All abolished production of OmpK36 upon exposure to lethal doses of imipenem and became highly imipenem resistant. After one week of daily drug-free passage, strains BR14, BR21 and BR28 (which we have termed non-reverters) retained high-level imipenem resistance through the permanent loss of OmpK36. BR7 and BR23 on the other hand, reverted to their original heteroresistance phenotype and resumed production of OmpK36. Strains BR7 and BR23 had IS*Ecp1*-mediated insertion of additional genes into the *ompK35* promoter region. This translocation in BR7 mediated major remodeling of the region. We have not yet tested whether reciprocal expression of *ompK35* is involved in the decline in production of OmpK36 upon imipenem exposure. If so, it is possible that the genetic alterations caused by IS*Ecp1* remodeling in BR7 and BR23 may be connected to their inability to permanently abolish OmpK36 production in order to retain high-level resistance. In our previous study of the KPC-producing *K. pneumoniae* strains, we did not detect the OmpK35 protein, but it is possible that our outer membrane extraction method was not sufficiently sensitive to detect the small amount of this protein that may be produced by these strains (1). We predominantly performed our tests with high-osmolarity media and we did not perform quantitative *ompK35* expression analysis. We cannot rule out the possibility of low levels of OmpK35 production, or the possibility of its production under specific environmental conditions.

The discovery of additional putative OmpR binding sites should also be explored further. The canonical OmpR binding sites are located adjacent to each other and OmpR~P is reported to cooperatively bind these sites as dimers (7). It is unknown whether an isolated binding site can effectively bind OmpR~P with high affinity, and our laboratory plans to test this possibility. The potential presence of additional OmpR binding sites and IS*Ecp1* in the *ompK35* promoter region

provide intriguing possibilities for another aspect of reciprocal regulation of *ompK35* and *ompK36.*

We also sought to uncover differences between the imipenem heteroresistant *K. pneumoniae* and *E. cloacae* study strains and the more highly resistant strains, *K. pneumoniae* BR1, BR3, CBpB3 and *E. coli* BR2. All of the latter, with the exception of BR3, had some of the characteristics of heteroresistance including the presence of colonies in the zone of inhibition of imipenem ETEST®. These three strains also had a 4-fold increase in imipenem MICs when tested in 10^6 cfu/ml starting inocula as compared to the $5x10^5$ cfu/ml reference standard inocula. While not as pronounced as the heteroresistant *K. pneumoniae* study strains, the increase is much greater than that observed in non-KPC-producing control strains. The more resistant strains (BR2, BR1, CBpB3) had IS*Kpn6*-mediated rearrangements of the KPC-containing Tn*4401* structure. No change in bla_{KPC} expression was detected by real-time reverse transcription PCR analysis of the heteroresistant *K. pneumoniae* study strains (1), however this analysis has not yet been performed with the resistant strains. Neither BR2 nor CBpB3 abolished production of OmpK36 (OmpC in BR2) during lethal imipenem exposure, while the heteroresistant *K. pneumoniae* study strains and *E. cloacae* BR10 did so. However, the nucleotide sequence of *ompC* in BR2, and *ompK36* in CB49pB3 had only 80% and 94% identity, respectively, to the heteroresistant *K. pneumoniae* and resistant BR1 and BR3 study strains. Thus BR2 and CBpB3 may produce a porin with a channel that is less permissive for imipenem entry as we found with imipenem-resistant strain *K. pneumoniae* BR20 in our previous study (1). This strain had colonies crowding the entire zone of inhibition of imipenem ETEST®, but the genetic arrangement of its Tn*4401* structure is currently unknown. While neither BR1 nor BR3 produced OmpK36 during imipenem exposure or in drug-free conditions, BR1 produced an alternate porin in drug-free conditions, which was subsequently abolished during exposure to imipenem. This may account for the initial difference in imipenem resistance observed between these 2 strains. These data confirm the important role of porin production in the induction of high-level imipenem resistance. However, the role of bla_{KPC}, and perhaps of other genes within the Tn*4401* structure, need further elucidation.

REFERENCES

1. **Adams-Sapper S, Nolen S, Donzelli GF, Lal M, Chen K, Justo da Silva LH, Moreira BM, Riley LW.** 2015. Rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing Klebsiella pneumoniae. Antimicrob Agents Chemother doi:10.1128/AAC.05100-14.
2. **Carlone GM, Thomas ML, Rumschlag HS, Sottnek FO.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from Haemophilus species. J Clin Microbiol **24:**330-332.
3. **Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, Edwards RA, Formsma K, Gerdes S, Glass EM, Kubal M, Meyer F, Olsen GJ, Olson R, Osterman AL, Overbeek RA, McNeil LK, Paarmann D, Paczian T, Parrello B, Pusch GD, Reich C, Stevens R, Vassieva O, Vonstein V, Wilke A, Zagnitko O.** 2008. The RAST Server: rapid annotations using subsystems technology. BMC Genomics **9:**75.
4. **Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, Moller Aarestrup F, Hasman H.** 2014. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother **58:**3895-3903.
5. **Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, Aarestrup FM, Larsen MV.** 2012. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother **67:**2640-2644.
6. **Chen L, Chavda KD, Fraimow HS, Mediavilla JR, Melano RG, Jacobs MR, Bonomo RA, Kreiswirth BN.** 2013. Complete nucleotide sequences of blaKPC-4- and blaKPC-5-harboring IncN and IncX plasmids from Klebsiella pneumoniae strains isolated in New Jersey. Antimicrob Agents Chemother **57:**269-276.
7. **Yoshida T, Qin L, Egger LA, Inouye M.** 2006. Transcription regulation of ompF and ompC by a single transcription factor, OmpR. J Biol Chem **281:**17114-17123.
8. **Prilipov A, Phale PS, Koebnik R, Widmer C, Rosenbusch JP.** 1998. Identification and characterization of two quiescent porin genes, nmpC and ompN, in Escherichia coli BE. J Bacteriol **180:**3388-3392.
9. **Landman D, Bratu S, Quale J.** 2009. Contribution of OmpK36 to carbapenem susceptibility in KPC-producing Klebsiella pneumoniae. J Med Microbiol **58:**1303-1308.
10. **Elliott E, Brink AJ, van Greune J, Els Z, Woodford N, Turton J, Warner M, Livermore DM.** 2006. In vivo development of ertapenem resistance in a patient with pneumonia caused by Klebsiella pneumoniae with an extended-spectrum beta-lactamase. Clin Infect Dis **42:**e95-98.
11. **Mena A, Plasencia V, Garcia L, Hidalgo O, Ayestaran JI, Alberti S, Borrell N, Perez JL, Oliver A.** 2006. Characterization of a large outbreak by CTX-M-1-producing Klebsiella pneumoniae and mechanisms leading to in vivo carbapenem resistance development. J Clin Microbiol **44:**2831-2837.
12. **Poirel L, Heritier C, Spicq C, Nordmann P.** 2004. In vivo acquisition of high-level resistance to imipenem in Escherichia coli. J Clin Microbiol **42:**3831-3833.
13. **Song W, Suh B, Choi JY, Jeong SH, Jeon EH, Lee YK, Hong SG, Lee K.** 2009. In vivo selection of carbapenem-resistant Klebsiella pneumoniae by OmpK36 loss during meropenem treatment. Diagn Microbiol Infect Dis **65:**447-449.

14. **Thiolas A, Bollet C, La Scola B, Raoult D, Pages JM.** 2005. Successive emergence of Enterobacter aerogenes strains resistant to imipenem and colistin in a patient. Antimicrob Agents Chemother **49:**1354-1358.
15. **Tsai YK, Fung CP, Lin JC, Chen JH, Chang FY, Chen TL, Siu LK.** 2011. Klebsiella pneumoniae outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. Antimicrob Agents Chemother **55:**1485-1493.
16. **Tsai YK, Liou CH, Fung CP, Lin JC, Siu LK.** 2013. Single or in combination antimicrobial resistance mechanisms of Klebsiella pneumoniae contribute to varied susceptibility to different carbapenems. PLoS One **8:**e79640.
17. **Naas T, Cuzon G, Truong HV, Nordmann P.** 2012. Role of ISKpn7 and deletions in blaKPC gene expression. Antimicrob Agents Chemother **56:**4753-4759.
18. **Bryant KA, Van Schooneveld TC, Thapa I, Bastola D, Williams LO, Safranek TJ, Hinrichs SH, Rupp ME, Fey PD.** 2013. KPC-4 Is encoded within a truncated Tn4401 in an IncL/M plasmid, pNE1280, isolated from Enterobacter cloacae and Serratia marcescens. Antimicrob Agents Chemother **57:**37-41.
19. **Gomez SA, Pasteran FG, Faccone D, Tijet N, Rapoport M, Lucero C, Lastovetska O, Albornoz E, Galas M, Group KPC, Melano RG, Corso A, Petroni A.** 2011. Clonal dissemination of Klebsiella pneumoniae ST258 harbouring KPC-2 in Argentina. Clin Microbiol Infect **17:**1520-1524.
20. **Naas T, Bonnin RA, Cuzon G, Villegas MV, Nordmann P.** 2013. Complete sequence of two KPC-harbouring plasmids from Pseudomonas aeruginosa. J Antimicrob Chemother **68:**1757-1762.
21. **Delihas N, Forst S.** 2001. MicF: an antisense RNA gene involved in response of Escherichia coli to global stress factors. J Mol Biol **313:**1-12.
22. **Liu X, Ferenci T.** 2001. An analysis of multifactorial influences on the transcriptional control of ompF and ompC porin expression under nutrient limitation. Microbiology **147:**2981-2989.
23. **Liu X, Ferenci T.** 1998. Regulation of porin-mediated outer membrane permeability by nutrient limitation in Escherichia coli. J Bacteriol **180:**3917-3922.
24. **Taylor RK, Garrett S, Sodergren E, Silhavy TJ.** 1985. Mutations that define the promoter of ompF, a gene specifying a major outer membrane porin protein. J Bacteriol **162:**1054-1060.
25. **Chen S, Zhang A, Blyn LB, Storz G.** 2004. MicC, a second small-RNA regulator of Omp protein expression in Escherichia coli. J Bacteriol **186:**6689-6697.
26. **Inokuchi K, Furukawa H, Nakamura K, Mizushima S.** 1984. Characterization by deletion mutagenesis in vitro of the promoter region of ompF, a positively regulated gene of Escherichia coli. J Mol Biol **178:**653-668.
27. **Mizuno T, Mizushima S.** 1986. Characterization by deletion and localized mutagenesis in vitro of the promoter region of the Escherichia coli ompC gene and importance of the upstream DNA domain in positive regulation by the OmpR protein. J Bacteriol **168:**86-95.
28. **Ramani N, Hedeshian M, Freundlich M.** 1994. micF antisense RNA has a major role in osmoregulation of OmpF in Escherichia coli. J Bacteriol **176:**5005-5010.
29. **Cai SJ, Inouye M.** 2002. EnvZ-OmpR interaction and osmoregulation in Escherichia coli. J Biol Chem **277:**24155-24161.

30. **Hernandez-Alles S, Alberti S, Alvarez D, Domenech-Sanchez A, Martinez-Martinez L, Gil J, Tomas JM, Benedi VJ.** 1999. Porin expression in clinical isolates of Klebsiella pneumoniae. Microbiology **145 (Pt 3):**673-679.
31. **Poirel L, Decousser JW, Nordmann P.** 2003. Insertion sequence ISEcp1B is involved in expression and mobilization of a bla(CTX-M) beta-lactamase gene. Antimicrob Agents Chemother **47:**2938-2945.
32. **Cao V, Lambert T, Courvalin P.** 2002. ColE1-like plasmid pIP843 of Klebsiella pneumoniae encoding extended-spectrum beta-lactamase CTX-M-17. Antimicrob Agents Chemother **46:**1212-1217.
33. **Wang Y, Song C, Duan G, Zhu J, Yang H, Xi Y, Fan Q.** 2013. Transposition of ISEcp1 modulates blaCTX-M-55-mediated Shigella flexneri resistance to cefalothin. Int J Antimicrob Agents **42:**507-512.

Table 6. Characteristics and imipenem susceptibility for KPC-producing Enterobacteriaceae strains and controls used in the study described in Chapter Two.

			IPM MIC (ug/ml)					
Strain[1]	ST	INFECTION SOURCE	$5x10^5$ (ref)[2]	$5x10^6$	$5x10^7$	$5x10^8$	ETEST ®[3]	SPECIES
BR7 (HET)	437	Blood	1-2	16	>32	>32	1+	*K. pneum.*
BR14(HET)	437	Blood	2	16	>32	>32	1+	*K. pneum.*
BR14ΔK36 (RES)	437	*ompK36* mutant of BR14	>32	>32	>32	>32	3+	*K. pneum.*
BR21 (HET)	437	Urine	1-2	16	>32	>32	1+	*K. pneum.*
BR23 (HET)	437	Blood	2	16	>32	>32	1+	*K. pneum.*
BR28 (HET)	483	Rectal swab	2	16	>32	>32	1+	*K. pneum.*
BR1 (HET/RES)	340	Rectal swab	2-16	16-32	>32	>32	2+	*K. pneum.*
BR3 (RES)	340	Rectal swab	>32	>32	>32	>32	3+	*K. pneum.*
BR2 (HET/RES)	20	Rectal swab	4	16	32	>32	1+	*E. coli*
BR10 (HET)	NT	Blood	2	4-8	>32	>32	1+	*E. cloacae*
CB49pB3 (RES)	483	Study transformant of CB49	4	16	32	>32	2+	*K. pneum.*
CB49 (SUSC)	483	Urine	0.25	0.25	0.25	0.25	0	*K. pneum.*
SF519 (SUSC)	66	Blood	0.25	0.25	0.5	4	0	*K. pneum.*

[1] Study strain sources: BR, CB, 6 hospitals, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil; SF, San Francisco General Hospital, San Francisco, CA, USA; HET: IPM heteroresistant phenotype; RES: high-level IPM resistance; HET/RES: characteristics of IPM heteroresistance (inoculum-dependent IPM increase and colonies growing in the zone of inhibition of IPM ETEST®, but exhibit higher reference standard IPM MICs than the defined heteroresistant strains in this study; SUSC: IPM susceptible control strain.

[2] CLSI reference standard inoculum.

[3] Number of colonies within zone of inhibition with ETEST®: 0, no colonies; 1+, <50 colonies (within lower region of zone); 2+, >50 colonies (within entire zone); 3+, no zone of inhibition.

ST: Multilocus Sequence Type; IPM: imipenem; NT: not typed.

Table 7. *bla*$_{KPC}$, and other β-lactamase and drug-resistance genes present on plasmids.

Plasmid name	pBR7	pBR21	pBR28	pBR3	pBR1	pBR14[1]	pBR23	CB49pB3	pBR10	pBR2
bla$_{KPC-2}$	√	√	√	√	√	√	√	√	√	√
bla$_{CTX-M}$ types	15	-	-	-	-[2]	9	15	8	9	-
dfr types	A30	A30	A30	A12	A12	A30	A30	-	A16	-
aad types (AMG)	A2	A2	A2	-	A2	-	-	A1	A2,B	-
aph types (AMG)	-	(3')1a	(3')1a	(3')1a	(3')1a	-	(3')1a	-		-
aac(3') types (AMG)	IId	1b-cr	-	-	-	1q, IIa	IId, Ib-cr	1b-cr	1b-cr, A4	-
qnr types (QUIN)	B1	-	B1	-	-	B1, A1	B1	-	A16	-
cat types (CAM)	A1	B3	A1	-	-	A1	A1, B3	-	-	-
bla$_{TEM}$ types	1b	-	-	-	-	1a/1b	1b	-	1a	-
bla$_{SHV}$ types[3]	11	11	11	11	11	11	11	-	12	-
mphA (MCL)	-	√	√	√	√	√	√	-	-	-
tet types (TET)	-	A	A	-	-	A, B	A, D	-	-	-

[1] Strain BR14ΔK36 is100% identical to BR14

[2] Strain BR1 was previously reported positive for *bla*$_{CTX-M}$ by PCR (1), but was negative by whole genome sequencing.

[3] *bla*$_{SHV}$ is located on the chromosome in these strains.

AMG, aminoglycoside; QUIN, quinolone; CAM, chloramphenicol; MCL, macrolide; TET, tetracycline; √, present; -, not present.

Table 8. Comparison of select characteristics of imipenem heteroresistant and resistant study strains.

Strain	BR7	BR21	BR10	BR2	BR1	BR3	CBpB3
% ID to *ompK36*[1]	100	100	79 (*omp36)*	80 (*ompC)*	100	100	94
ompK35/IS[2]	trans	un	un	un	interr	interr	no IS
Tn4401 arrangement[3]	none	none	insertion, IS*Kpn6*	trans, IS*Kpn6*	trans, IS*Kpn6*	trans, IS*Kpn6*	trans, IS*Kpn6*
↓OmpK36/IPM[4]	yes	yes	yes	no	yes	yes	no

[1] Percent identity to GenBank accession no. JX310551 (homolog of *ompK36,* if relevant).

[2] Interruptions (interr) or translocations (trans) mediated by insertion elements or hypothetical proteins present in *ompK35* regulatory region (or homologs): BR7, BR21 (IS*Ecp1*); BR10, BR2 (hypothetical protein); BR1, BR3 (IS*1*); un, uninterrupted; no IS, no elements present.

[3] IS*Kpn6*-mediated rearrangements of Tn*4401* (see text and Figure 3): ins: insertion of genes within IS*Kpn6*; trans, translocation of portion of IS*Kpn6* and insertion of *rep*-like, *rom*, *excl1* genes.

[4] yes: strain abolished production of OmpK36 (or homolog) upon exposure to lethal concentration of imipenem. Note that BR1 and BR3 also did not produce OmpK36 in drug-free conditions (see text).

Table 9. Characteristics of plasmids harbored by study strains. Replicon types and the mosaic composition of the plasmids are shown.

Plasmid name	pBR7	pBR21	pBR28	pBR3	pBR1	pBR14[1]	pBR23	CB49 pB3	pBR10	pBR2
Species	*Kp*	*Kp*	*Kp*	*Kp*	*Kp*	*Kp*	*Kp*	*Kp*	*Eclo*	*Ec*
IPM phenotype	HET	HET	HET	HET-RES	HET	HET	HET	RES	HET	HET-RES
plasmid rep origins[2]										
pQIL (FIB)	√	√	√	-	-	-	√	-	-	-
FIB	-	K	K	K	K	mar	K	K (98.9)[2]	-	*Ec*
FII	-	K	K	K	K	yp	K	K (95.9)[2]	-	*Ec*
N	-	√	√	√	√	√	√	-	-	√
HI	-	-	-	-	-	1B (mar)	-	-	2/2a	-
ColR-NAI	-	-	-	√	√	-	-	-	-	-
L/M	-	-	-	-	-	-	-	√	-	-
X3	-	-	-	-	-	-	-	-	√	-
FIA	-	-	-	-	-	-	-	-	-	*Ec*
assoc of bla_{KPC-2}	pQIL	N	N	N	N	N	N	TBD[3]	X3	N
Common regions of alignment (GenBank accession no., species, plasmid name, nt range)										
IncFIB, FII region										
CP010390, *K. pneum.*, p6234.198										
53832-56665	√	√	√	-	-	-	√	-	-	-
92827-104299;107122-109002	-	√	8kb	-	-	-	3kb	-	-	-
CP00662, *K.pneum.*, ATCC BAA-2146, pHg										
29785-32882	-	-	√	-	-	-	√	-	-	-
44174-48457	√	-	-	-	-	-	√	-	-	-
U7194.4, *E. aerogenes* R751										
35101-37729	-	√	√	-	-	-	√	-	-	-
31708-33062	√	√	√	-	-	-	√	-	-	-
CP000965, K. pneum. strain 342 pKp187										
112980-120088	√	√	√	-	-	-	√	-	-	-
JX424474, *K. pneum.*, pKPN-4 of MGH78578 (pQIL)										
60950-62550, IS*903b*[5]	√	-	-	-	-	-	√	-	-	-
29815-54088	√	√	√	-	-	-	√	5.8kb	-	-
CP009777, *K.pneum.*, KPNIH32-pKPNa68										
297-1651	√	√	√	√	√	√	√	-	-	-

2562-3353	√	√	√	√	√	-	-	-	-	-
3252-5002	-	√	√	√	√	-	√	-	-	-
6350-14444	-	√	√	√	√	-	√	-	-	-
18431-186707	-	√	√	√	√	-	√	63kb	-	-
186699-201670	-	√	√	√	√	-	√	-	-	-
206188-206955	√	√	-	-	-	-	-	-	-	-
IncN region										
CP004367, *K. pneum.*, FCF/3SP[3]										
region1: resP, secA,	-	√	√	√	√	√	√	-	-	√
region2: repA(IncN), to Δnuc Tn4401 insertion	(from part of *traI*)	√	√	√	√	√	√	-	-	√
region3: Tn4401	√	√	√	√[4]	√[4]	√	√	√[4]	√[4]	√
Region 4: traG to EcoRII	√	√	√	√	√	√	√	-	to *traE*	√
HQ840942. *S. enterica*, serovar Typhimurium pSRC27-H[6]										
10112-11475;14719-17068;25721-32737	√	-	-	-	-	-	√	-	-	-

[1] Strain BR14ΔK36 is100% identical to BR14

[2] NCBI references for plasmid origins of replication (Inc types; 100% identity unless noted): FIB(pQIL), JN233705; FIB(K), JN233704, 100% identity except CB49pB3; FII(K), CP000648, 100% identity except CB49pB3; FIB (mar), JN420336; FII(yp), CP00067; N, AY046276, 99.4% identity; *Ec*, AY458016 (FII), and AP0011918 (FIB, FIA); HI1B, JN420336; X3, JN247852, H12/2a, BX664015; L/M, U27345, 99.4% identity; ColRNAI, DQ298019.

[3] CP004367 nucleotide regions of alignment in chart: region 1, 352-4908; region 2, 5474-26931; region 3, 27104-37551; region 4, 37648-54446.

[4] These strains have rearrangements of the Tn*4401* structure. See text and Figure 3.

[5] Interrupts IS*Ecp1* upstream of $bla_{\text{CTX-M-15}}$ in these strains; 100% identical to element following Tn*4401* structure.

[6] Present on the chromosome in these strains.

√, present in strain; -, not present in strain. *Kp, K. pneumoniae; Eclo, E. cloacae, Ec, E. coli*; HET, heteroresistant, RES, resistant; IPM, imipenem.

Table 10. Putative OmpR regulatory binding sites in *ompK35* and *ompK36* genetic regions.

ompK region/binding site	canonical[1]	CRIT[2] a/b	*Kp* CB49[3]	CRIT (n)[2] a/b	*Kp*-KPC[4]	CRIT (n)[2] a/b
ompK35:						
F4	**G**TT**AC**GGAAT ATT**ACA**TTGC	3/3	**G**TT**AC**GCACT GTT**T**C**G**GTGC	3/1	**G**TT**AC**GCACT GTT**T**C**G**GTGC	3/1
F1	TTT**AC**TTTTG **G**TT**ACA**TATT	2/4	TAC**AC**ATTTA **G**TT**ACA**TGAT	2/4	TAC**AC**ATTTA **G**TT**ACA**TGAT	2/4
F2	TTTT**C**T**T**TTT **G**AA**AC**CAAAT	2/3	TTTT**C**G**T**TTT **G**TT**AC**TCATA	2/3	TTTT**C**G**T**TTT **G**TT**AC**TCATA	2/3
F3	CTTTATCTTT **G**TAG**CA**C**T**TT	0/4	TGATATATTC **G**TAG**CA**T**T**TT	-/4	TGAGATATTC **G**TAG**CA**T**T**TT	-/4
F2 alt[5]	TTTT**C**T**T**TTT **G**AA**AC**CAAAT	2/3	None	-	TTTT**A**T**T**TTT **G**AA**A**AGAAAC	1/2
F3 alt[5]	CTTTATCTTT **G**TAG**CA**C**T**TT	0/4	None	-	CTTTTTCTTT **G**TA**CA**A**A**TA	-/3
F2 alt 2[5]	TTTT**C**T**T**TTT **G**AA**AC**CAAAT	2/3	None	-	TCTT**C**C**T**CAA **G**CG**AC**TTCTT	1/3
ompK36:						
C1	TTT**ACAT**TTT **G**AA**ACAT**CTA	4/5	TTT**ACA**A**A**TT **G**AA**ACAT**CTT	3/5	TTT**ACA**A**A**TT **G**AA**ACAT**CTT	3/5
C2	AGCGATAAAT **G**AA**ACAT**CTT	0/5	TGGGAACTTT **G**AA**ACAT**CTT	-/5	TGGGAACTTT **G**AA**ACAT**CTT	-/5
C3	AAAAGTTTTA **G**TAT**CAT**ATT	0/4	AGAAGTTTTA **G**TAT**CAT**ATT	-/4	AGAAGTTTTA **G**TAT**CAT**ATT	-/4

[1] Canonical phosphorylated ompR (ompR~P) binding sites as determined by Yoshida et al.(7).

[2] CRIT: number of residues determined by Yoshida et al. to be critical for high affinity binding (bold and underlined), and number of those sites present in study strains. Number of CRIT sites reported separately for "a" sites (1st 10 nucleotides) and "b" sites (nucleotides 11-20); b site residues were found to be more critical than a sites.

[3] *K. pneumoniae* strain used as the prototypical strain containing OmpR~P binding sites highly similar to canonical sites. Mismatched sites (SNPs) shown in red. Data not shown: putative binding sites found in *E. coli* BR2 (100% identity to all canonical sites), and *E. cloacae* BR10 (not 100% identical, but contained all critical binding residues); BR1 and BR3 had no such sites due to insertion sequence.

[4] These sites were found in *K. pneumoniae* strains (BR7, BR14, BR14ΔK36, BR21, BR23, BR28). SNPs shown in red.

[5] These sites were identified in this study as potential additional OmpR~P binding sites created by IS*Ecp1* insertion (F2 alt, F3 alt), and *orfB* insertion (F2 alt 2) elements.

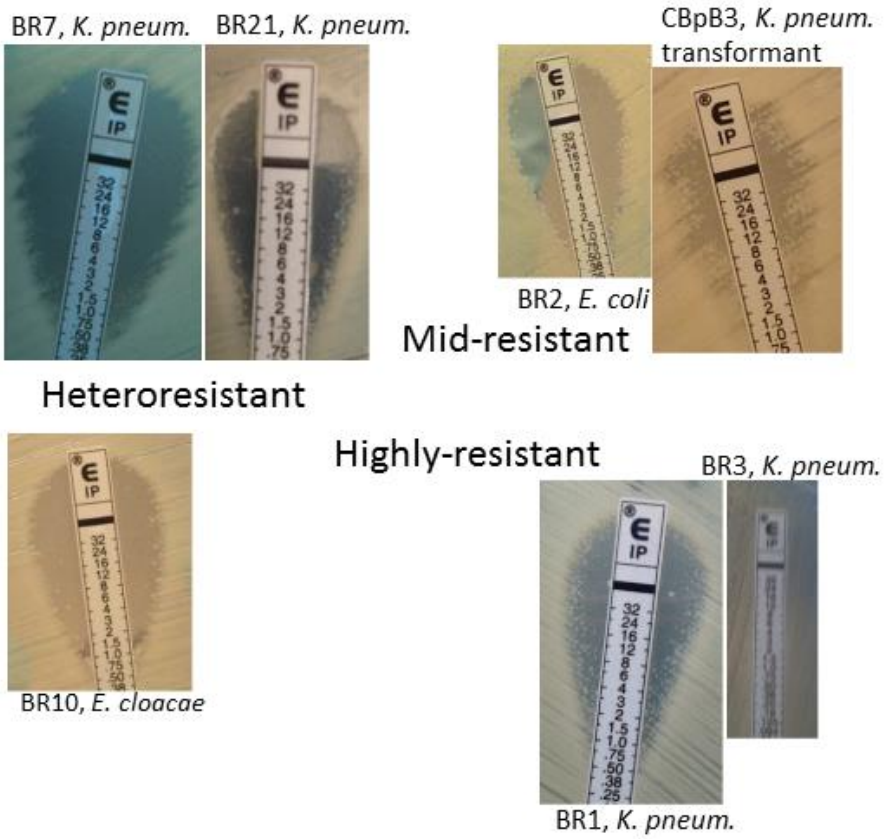

Figure 2. Characteristics of phenotypic heteroresistance by imipenem ETEST® (colonies in the zone of inhibition) in strains with imipenem resistance levels as determined by reference standard MIC testing. Note the paradoxical susceptibility of strains BR1 and BR2 compared to imipenem broth microdilution in Table 6. CBpB3 was transformed with the bla_{KPC}-containing plasmid from highly-resistant *K. pneumoniae* BR3 (no zone of inhibition).

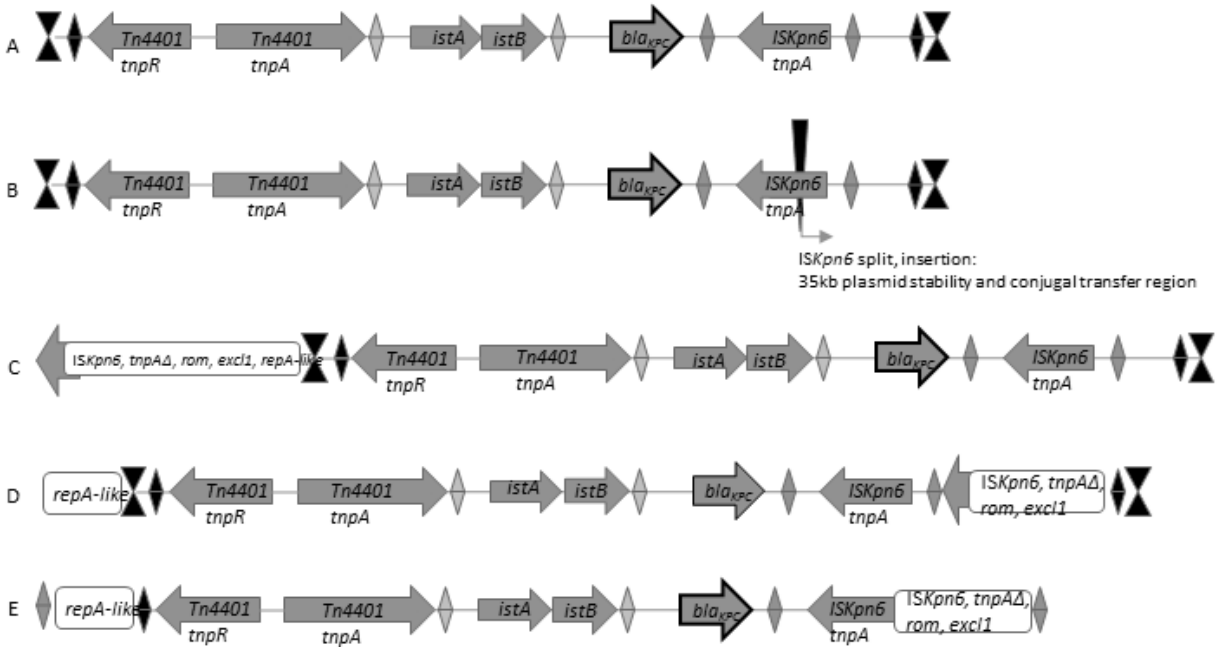

Figure 3. Tn*4401* and rearrangements in the study strains. Typical structure of Tn*4401*, present in heteroresistant *K. pneumoniae* BR7, BR14, BR21, BR23, BR28 (A); IS*Kpn6*-mediated rearrangement of Tn*4401* in heteroresistant *E. cloacae* BR10 (B), and in resistant strains *K. pneumoniae* BR1, BR3 (C), *E. coli* BR2 (D), and the CBpB3 transformant (E).

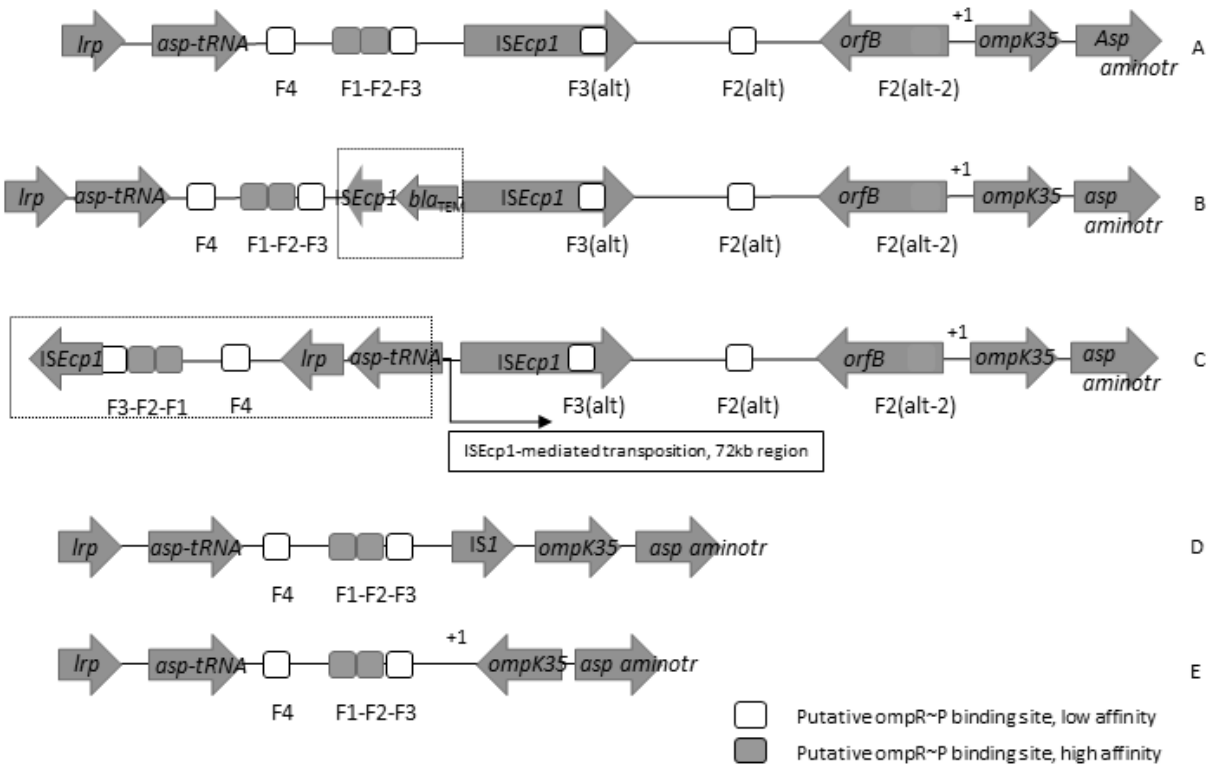

Figure 4. *ompK35* genetic region in the study strains. A. Heteroresistant *K. pneumoniae* strains BR14, BR21, BR28 (non-reverters); B. Heteroresistant *K. pneumoniae* BR23 (reverter); C. Heteroresistant *K. pneumoniae* BR7 (reverter); D. *K. pneumoniae* highly resistant strains BR1, BR3; E. Mid-resistant strains *E. coli* BR2, *K. pneumoniae* transformant CBpB3, heteroresistant strain *E. cloacae* BR10 (not shown: 314bp hypothetical protein, 75nt from *omp35* coding region, with 99% identity to a portion of a Gram-negative porin (GenBank accession no. CP003737).

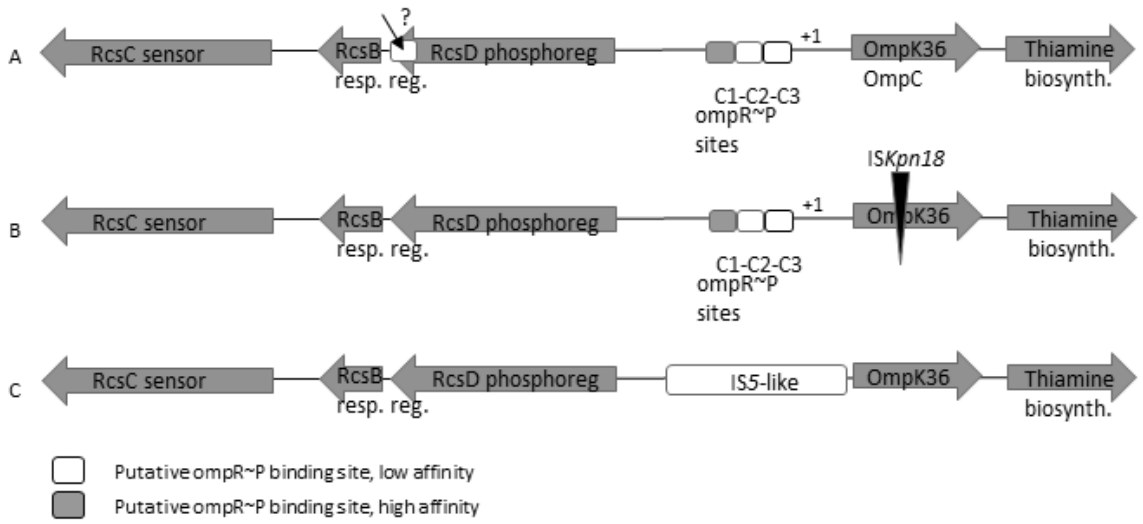

Figure 5. *ompK36* genetic region in the study strains. A. Present in all study strains with the exception of: resistant strain *K. pneumoniae* BR14ΔK36, interruption of *ompK36* (B), and *K. pneumoniae* BR1, and BR3, disruption of OmpR binding sites (C).

CHAPTER THREE. Coordinated mechanisms mediate expression of carbapenem heteroresistance in KPC-producing *Klebsiella pneumoniae*.

INTRODUCTION

Carbapenem heteroresistant KPC-producing Enterobacteriaceae have highly similar heteroresistance phenotypes, yet are genetically diverse in their plasmid content other than carriage of the bla_{KPC}-containing Tn*4401*. The 9 heteroresistant strains we characterized in our past studies (*K. pneumoniae* BR6, BR7, BR14, BR19, BR21, BR23, BR26, BR28, and *E. cloacae* BR10) showed similar inoculum-dependent increases in imipenem resistance, had similar frequencies of heteroresistant subpopulations that could grow in lethal concentrations of imipenem 8- to 16-fold higher than their reference standard minimum inhibitory concentrations (MICs) of 1-2ug/ml, and could abolish production of OmpK36 (Omp36 in *E. cloacae*), with exposure to lethal doses of imipenem (1). We detected the presence of distinct subpopulations of cells (BR6, BR14, BR21, BR23, BR28), representing a range of resistance, in imipenem-exposed cultures derived from a single clonal population that was relatively susceptible to imipenem prior to exposure. Highly resistant colonies selected from this subpopulation did not produce OmpK36. We also detected more susceptible colonies in the subpopulation that produced OmpK36. We hypothesize that these heterogeneous populations arose from the small population of cells (0.1% or less) that initially survived imipenem-mediated killing. Although we did not detect OmpK36-deficient mutants among those initial survivors, we cannot rule out the possibility of their existence. However, the presence of a heterogeneously resistant subpopulation argues in favor of our hypothesis that the survivors possess specific metabolic characteristics that facilitate simultaneous tolerance to high concentrations of imipenem, while carrying out the necessary protein synthesis for drug inactivation, porin regulation and ultimate recovery.

We found two types of OmpK36 production in the *K. pneumoniae* strains (the *E. cloacae* strain has not yet been tested). BR6, BR14, BR19, BR21, and BR28 are members of what we have termed the non-reverter group. Abolished production of OmpK36 is permanent in these strains due to an insertion sequence in the coding region of *ompK36* and these strains retain high-level imipenem resistance. BR7 and BR23 are members of the reverter group. Upon exposure to lethal concentrations of imipenem, these strains stop producing OmpK36 without mutating the underlying gene, but revert to an imipenem heteroresistance phenotype and resume OmpK36 production when the drug is removed. We discovered differences in the promoter region of the *ompK35* gene between the strains in the reverter and non-reverter groups. IS*Ecp1* and *orfB* insertion elements were present in this region in all of the heteroresistant strains (with the exception of *E. cloacae* BR10), but in none of the mid- to highly resistant strains. The IS*Ecp1* element mediated a rearrangement in the *ompK35* region in the reverter strains BR7 and BR23. Consequently, we sought to detect the specific mechanisms that mediate *ompK36* expression in the absence of mutations. Since *ompK35* and *ompK36* are reciprocally regulated, the presence of the insertion elements in the *ompK35* region may have a role in the expression of the heteroresistance phenotype.

In this study we focused our analysis on host strain characteristics, starting with transposon mutagenesis of the reverter-type heteroresistant BR7 strain. We identified mutants with diminished or absent imipenem heteroresistance that also failed to abolish OmpK36 production. Our initial findings implicate the role of bacterial metabolism, showing that acquisition of specific nutrients at a critical point during lethal imipenem exposure is necessary for the coordination of porin regulation that mediates the expression of the heteroresistance phenotype.

METHODS

Strains and susceptibility testing. Two KPC-producing *K. pneumoniae* strains with an imipenem heteroresistance phenotype were included in this study. BR7 was included as a representative of the OmpK36 reverter group. BR21 is a representative of the OmpK36 non-reverter group. Mutant strains of BR7 derived from transposon mutagenesis experiments (described below) are shown in Figure 6. Five of these mutants (A1.2, B3.1, B3.2, E7, E11) were selected for the experiments described in this study.

Imipenem susceptibility testing. Antimicrobial susceptibility testing was performed by broth microdilution in accordance with the standards set by Clinical and Laboratory Standards Institute (CLSI) and ETEST® (bioMérieux, Marcy l'Etoile, France). Imipenem (Sigma-Aldrich, St. Louis, MO) was used as the representative carbapenem drug in all experiments. Inoculum-dependent increases in the MIC for imipenem were determined based on the CLSI reference standard starting inoculum of $5x10^5$ cfu/ml compared to inocula of $5x10^6$, $5x10^7$ and $5x10^8$cfu/ml. An inoculum effect was considered positive if the higher test inocula resulted in an 8-fold or greater increase in the imipenem MIC. Cation-adjusted Mueller Hinton broth (MH) was used in all experiments unless otherwise noted.

Analysis of outer membrane proteins. Outer membrane proteins were isolated according to the method of Carlone et al (2). Briefly, samples were grown in nutrient broth (NB) or MH to an OD_{600} of 0.6, centrifuged at 5000xg for 10 minutes (m), washed and resuspended in 10mM HEPES buffer, pH 7.4 and sonicated. The sodium N-lauroyl sarcosinate insoluble outer membrane porins were selectively obtained by incubation in 10mM HEPES buffer with 2% sarkosyl, followed by 30 m centrifugation at 15,600xg. Samples were boiled and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gels (Bio-Rad, Hercules, CA). Controls included drug-susceptible *Klebsiella pneumoniae* strains and previously characterized OmpK36 mutant strains (1).

Transposon mutagenesis, identification of insertion site and gene mutations. Transposon mutagenesis was performed with the EZ-Tn5™ <KAN-2> Tnp Transposome™ kit (Epicentre Biotechnologies, Madison, WI) according to the manufacturer's protocols. Mutants selected from kanamycin selective plates were subjected to a screen to select those with diminished or abolished expression of imipenem resistance relative to wild-type BR7. Samples of overnight cultures of mutant strains were diluted to achieve a 10^6 cfu/ml starting inoculum, then grown in the presence of 2 and 8ug/ml imipenem for 18-20 hours (h). Samples that could grow in the lower, but not the higher, imipenem concentration were selected for further analysis. Inoculum-dependent imipenem MICs were assessed for the selected mutants as described above.

The site of transposition was identified with a 3-step PCR protocol (personal communication, Sangwei Lu, Lu Lab, University of California, Berkeley). The bacterial DNA was extracted from overnight cultures by spinning at maximum speed for 2 m, removal of the supernatant and resuspension of the pellet in 100µl of PCR grade water. Samples were boiled for 10 m, then spun at maximum speed for 15 m. For the first PCR round, 1µl of the supernatant was used as template DNA in a 50µl reaction containing 0.2 mM deoxynucleoside triphosphates (dNTPs), 0.5µl of Invitrogen *Taq* polymerase, 1x Invitrogen PCR buffer, and 0.25µM of one primer (Inv-1, Inv-2 run in separate reactions). 1st round PCR products were ethanol precipitated, resuspended in 17µl PCR grade water with 1µl DpnI, then incubated at 37°C for 2 h. Following enzyme inactivation at 65°C for 20 m, 1µl of the digested DNA was used for 2nd round PCR in 25µl reactions with the reagents listed above. One µl of the 2nd round PCR product was used as template for the 3rd round PCR in 50µl reactions with the reagents listed above. The following program was used for 1st and 3rd round PCR: 35 cycles of 94°C, 30 s, 52°C, 30 s, 72°C, 1.5 m. The program for 2nd round PCR was the same, except the annealing temperature was 30°C. All PCR reactions were performed with the INV-1 (5'-ATGGCTCATAACACCCCTTGTATTA) or INV-2 (5'-GAACTTTTGCTGAGTTGAAGGATCA) primers listed in the EZ-Tn5™ kit. The final 3rd round PCR products were purified and sent for sequencing with the primers provided in the EZ-Tn5™ kit. Sequencing was performed on an Applied Biosystems 3730 DNA analyzer (Applied Biosystems, Foster City, CA) at the University of California Berkeley DNA Sequencing Facility. We inspected the DNA sequences with BioEdit version 7.0.1 to locate the mosaic ends (ME) of the Tn5 sequence. The sequence following the ME was submitted for a BLAST® search of the National Center for Biotechnology Information (NCBI) database. Information for the disrupted genes was obtained by submitting sequences to the Transporter Classification Database (3) and to the NCBI Conserved Domains Database (4).

Metabolite-potentiated killing assay. We performed glucose-mediated killing assays modified from those reported by Allison et al (5). Heteroresistant strain BR21 was exposed to 16ug/ml imipenem for 2 h to enrich for potential persisters. Cultures were spun, washed, and resuspended in the following: minimal media with 10mm glucose, and 16ug/ml imipenem or 0.5ug/ml gentamicin; Mueller-Hinton (MH) broth with 16ug/ml imipenem or 0.5ug/ml gentamicin. Control samples which were not pretreated with imipenem, but otherwise prepared as above, were used as controls. Experiments were repeated at least three times.

Growth rate assay. Overnight cultures were diluted to achieve a starting inoculum approximating $3x10^6$cfu/ml, which was confirmed by plating. Three replicates of each sample were grown in drug-free MH for 2 h, then plated on drug-free agar for enumeration. Generation time for each sample was calculated with the formula: growth time (in m) / (log (T_2) –log (T_0) / 0.301), where T_2 is the cfu/ml after 2 h of growth, and T_0 is the cfu/ml of the starting cultures.

Growth and imipenem resistance in varied nutrient and osmolarity conditions. Growth in imipenem was tested in the following media: full strength MH (high osmolarity, 300mOsm/L, high in peptide nutrients); dilute strength MH (6% MH, 94% minimal salts media, mid-level osmolarity, 200mOsm/L, low in peptide nutrients); NB (low osmolarity, ≤100mOsm/L, lower

than MH in peptide nutrients); NB with 10mm glucose (low osmolarity, ≤100mOsm/L, mixed nutrient source); minimal salts media with 10mm glucose (mid-level osmolarity, 200mOsm/L, glucose as a sole carbon source). A 10^6 cfu/ml starting inocula was prepared for each sample, then dispensed into wells with the above media to perform imipenem broth microdilution tests.

Population analysis. Population analysis was performed as previously described, with 10^7 bacterial cfu spread on imipenem-containing MH agar plates (1). We calculated the frequency of heteroresistant subpopulations at the highest drug concentrations after 24 h of growth by dividing the number of colonies grown on imipenem-containing plates by the colony counts from the same bacterial inoculum plated onto drug-free MH agar plates.

Density analysis. One ml cultures of 10^7 bacterial cfu were prepared, then dispensed into 20, 40, or 100 wells of a 96-well plate containing 16ug/ml imipenem (200ul total volume). One ml cultures of 10^8 bacterial cfu were prepared in order to dispense 10^7 bacterial cfu into 10 control wells. The samples were incubated at 37°C for 18-20 h and the number of wells with positive growth was recorded in each group. The frequency of heteroresistant subpopulation in a lethal dose of imipenem assumed a minimum of 1 cell per well mediated recovery of that population and was calculated as the number of positive wells divided by the total inoculum in 1ml of starting culture.

RESULTS

Phenotypic characteristics of mutants. We performed two transposon mutagenesis experiments with the heteroresistant *K. pneumoniae* KPC-producing strain BR7. This strain abolishes production of OmpK36 when exposed to lethal doses of imipenem, but the regulation is reversible. The porin becomes produced again upon removal of the drug exposure. We initially selected 14 mutants based on the results of our screen with two concentrations of imipenem (Figure 6). The typical heteroresistance phenotype observed in previous studies demonstrates an inoculum-dependent increase in the imipenem MIC for starting inocula from $5x10^5$ cfu/ml to $5x10^6$ cfu/ml (1). The wild-type heteroresistant BR7 strain can grow in concentrations of imipenem up to 16ug/ml with higher inocula, but typically does not exceed growth past 1ug/ml with reference standard inocula. We grouped the 14 mutants into three phenotypes based on their patterns of growth in concentrations of imipenem from 0.5-32ug/ml (Figure 6). The group 1 mutants had increased imipenem susceptibility (maximum growth in 2-4ug/ml at the higher inocula), and insignificant inoculum effect between the two inocula tested. One fully susceptible group 1 mutant (B3.1) was found to be bla_{KPC} negative by PCR. The group 2 mutants could grow well in concentrations up to 2ug/ml imipenem, but in contrast to the group 1 mutants, group 2 mutants showed expression of heteroresistance up to 32ug/ml in higher inocula. In contrast to our wild-type heteroresistant strains, group 2 mutants expressed heteroresistance at the lower inocula as well. In both inocula, frequency appears to be lower than in wild-type strains as evidenced by the lack of continuity of growth in imipenem. Two mutants (B4.2 and B2.2) showed patterns of heteroresistance very similar to the wild-type strain, and comprised our third phenotype.

We performed a more complete test for inoculum-dependent increases in imipenem MICs for mutants A1.2, B3.1, B3.2, E7 and E11 (Table 11). As expected, strain B3.1 that apparently lost bla_{KPC} was completely susceptible at all inocula tested. The other 4 mutants were much less resistant at the 10^6 cfu/ml inocula than their wild-type counterparts, but were highly resistant at the 10^7 and 10^8 cfu/ml inocula.

Site of transposon insertion in mutants. Four of the group 1 mutants (A1.2, A4.2, B3.1, B1.2), 3 of the group 2 mutants (A2.2, A3.2, A5) and 2 of the group 3 mutants (B2.2, B4.2) had transposon insertions in an amino acid ATP-binding protein that is 1 of 3 genes in an amino acid ABC transporter cluster (Table 12). A search of the Transporter Classification Database and the NCBI Conserved Domains Database classified the proteins encoded by these genes as an ATP-binding polar amino acid transporter, a transmembrane permease subunit component, and an extracellular solute binding protein with a glutamine binding domain. Group 1 mutant B3.2 and group 2 mutant D8 had a transposon insertion in a gene annotated as a phage tail protein. We have not yet identified the insertion site in mutants E7, E11 or B4.1.

Differences in expression of heteroresistance due to bacterial metabolism. We were interested in whether we could reproduce the findings of Allison et al. in regard to metabolite-mediated eradication of persisters (5). Persisters are thought to have reduced rates of protein synthesis, yet aminoglycosides, which target ribosomes, have weak activity against persisters. These researchers found that gentamicin uptake and subsequent bacterial killing was mediated by the addition of metabolites involved in upper glycolysis. One heteroresistant strain (BR21) was used for the following experiments. After enriching for presumed persisters by exposing a starting inoculum of cells to a lethal dose of imipenem, the resulting survivors were tested for their ability to survive in minimal media with glucose, plus a dose of gentamicin slightly above the MIC (Table 13). None of the twelve experimental samples survived the gentamicin exposure in minimal media with glucose. However, 11 (92%) of the 12 samples that were pre-exposed to imipenem, but resuspended in MH survived. Of the samples not subjected to imipenem pre-exposure, 11 (92%) of 12 survived in minimal media supplemented with glucose and gentamicin, and all 12 survived in MH and gentamicin.

We wondered if we could replicate these results with imipenem exposure after pretreatment. None of the 21 samples tested could grow in 16ug/ml imipenem in minimal media with glucose after 2 h of imipenem pretreatment, while 10 (83%) of 12 of the samples could grow in the same concentration if resuspended in MH. Unlike the experiment with gentamicin, only 1 (0.6%) of the 18 samples not subjected to imipenem pre-treatment could grow in minimal media with glucose and 16ug/ml imipenem, while 12 (80%) of 15 samples could grow in MH with this imipenem concentration.

To probe the difference in these experiments we performed MIC testing for gentamicin and imipenem in both MH and minimal media with glucose (Table 14). The gentamicin MIC was unchanged with respect to starting inocula and growth media. However, the imipenem MICs were significantly lower for samples grown in minimal media with glucose and the heteroresistant phenotype was absent. Since the primary gene disruption in our mutants was

identified in amino acid transport, we wished to further explore the effect of nutrients on the expression of heteroresistance.

To assess whether the lower MICs in glucose media were specific to glucose we tested the imipenem MICs of the wild-type strain BR7, and mutants BR7ΔK36, A1.2, B3.1, B3.2, E7 and E11, grown in dilute MH (Table 15). We reasoned that we could test the strains in conditions of nutrient limitation without changing the carbon source. All strains showed reduced growth of ≥65% in this media and growth in imipenem did not exceed 2ug/ml in any of the strains at 10^6 cfu/ml inocula. The BR7ΔK36 strain was surprisingly similar to the other mutants tested, showing complete imipenem susceptibility in this media.

A low osmolarity environment seems to be favored in mutants in absence of nutrients. Since the expression of *ompK35* and *ompK36* is reciprocally regulated, we wished to test the difference in heteroresistance due to growth in media types which we varied in nutrients and osmolarity. Wild-type trains BR7 (reverter type) and BR21(non-reverter type), and mutants BR7ΔK36, BR21ΔK36, A1.2, B3.2, E7, and E11 were grown in MH (high-osmolarity), in dilute MH and in minimal media with glucose (mid-level osmolarity with varied carbon sources), and Nutrient Broth (NB) with and without supplementation with 10mm glucose (low-osmolarity) (Table 15). In addition to differing in osmolarity, NB contains less peptide nutrients than MH.

Wild-type strains BR7 and BR21 showed the highest expression of heteroresistance in MH followed by NB with glucose, and showed reduced heteroresistance in higher osmolarity conditions with lack of peptide-type nutrients. The imipenem-exposed BR7 phenotype was similar to that of the transposon mutants in all media types except NB with glucose supplementation. BR7ΔK36 also had a phenotype similar to the transposon mutants, except it could express heteroresistance in low osmolarity NB, but not if supplemented with glucose. The BR21ΔK36 mutant, with permanently absent production of OmpK36, had relatively unaffected resistance to imipenem in any of the tested media, with the exception of reduced growth in diluted MH. Mutant E7 showed no heteroresistance in any of the media types. Mutants B3.2 and A1.2 expressed heteroresistance in NB with glucose, but not in the other media types. Mutant E11 expressed heteroresistance in both MH and in NB with glucose.

Wild-type BR7 could abolish OmpK36 in the presence of imipenem in any of the media types with the exception of minimal media with glucose. OmpK36 was present in mutants A1.2, E7, B3.1, B3.2 and E11 when grown in MH with imipenem, which was tested at the highest concentration of drug at which they grew. In mutants A1.2. B3.2 and E11, OmpK36 was absent with imipenem exposure in NB with or without glucose, but was present in all other tested media. This indicates a difference in regulation of *ompK36* during imipenem exposure in these different types of media.

Growth rate differs in low nutrient conditions. Although growth in drug-free media differed according to the media used, wild-type BR7, BR21 and the tested mutants (B3.1, B3.2, E11, E7, A1.2) had similar levels of growth in each of these media (Table 15). Since these results were based on end-points of 18-20 hours of growth, we wished to analyze potential differences in the early time points after transferring the strains to fresh media. The drug-free growth rates for

wild-type BR7 and the B3.1 mutant were compared in full strength MH and MH diluted in minimal media. The starting inocula was $3x10^6$ cfu/ml to represent the inocula used in previous time kill experiments and in the higher inocula MIC tests. The generation time during the first two hours of growth was 45 – 53 minutes for the BR7 strain in MH, and 261-521 minutes in the diluted media. The generation time was 194 – 300 minutes for the B3.1 mutant in MH and showed a slight decline in the population in the diluted media. All samples achieved turbidity by 20 h of growth. Interestingly, the BR7 wild-type strain grown in low nutrient conditions showed a similar growth rate as the mutant in full strength media.

Growth rate, but not initial survival, differs in the presence of glucose. We assessed the difference between survivors after 2 h of lethal imipenem exposure in heteroresistant strain BR21 grown in MH or minimal media with glucose (Table 16). The average number of survivors in MH with 16ug/ml imipenem was $9x10^3$ cfu/ml while the average number of survivors in minimal media with glucose was $5x10^3$ cfu/ml. All 3 of the samples grown in MH achieved population recovery while none of the minimal media samples did so. We lowered the concentration of imipenem to 4ug/ml and retested. The average number of survivors in MH was $1x10^5$ cfu/ml, and all 3 achieved population recovery. The average number in minimal media was $8x10^4$ cfu/ml, yet only 1 of the 3 samples recovered even though the initial killing resulted in less than a 2-log decrease of the starting inoculum.

We analyzed the generation times of strain BR21 grown in MH or minimal media with glucose for the first two hours of growth in the drug-free samples from the above experiment. The generation time of the 2 samples grown in MH was 28 and 26 minutes, while that of the 2 samples grown in minimal media was 91 and 540 minutes.

We then analyzed the generation time of BR21 after pre-treatment for 2 h in 16ug/ml imipenem in either MH or minimal media with glucose. The number of pre-treated samples enumerated after 2h of subsequent drug-free growth was 1-log lower in samples exposed to imipenem in minimal media (Table 16). The 2 h drug-free growth rate after exposure ranged from 81 – 112 minutes for the samples grown in MH. The samples grown in minimal media showed no increase in the 2 h of drug-free growth following pre-treatment, although the samples showed turbidity by 18 h. These results show the different physiological state of imipenem-exposed survivors grown in varied nutritional environments.

Frequency of imipenem heteroresistance is reduced in mutants. The frequency of colonies that grew on imipenem agar in concentrations above their imipenem MICs were much lower for the tested BR7 mutants than for wild-type BR7. Imipenem-susceptible mutant B3.1 grew on a maximum of 4ug/ml imipenem agar with a frequency of $1x10^{-8}$ and on 2ug/ml imipenem agar with a frequency of $6x10^{-8}$ (4- and 2-fold higher than the imipenem MIC, respectively). The mutant B3.2 grew on 8ug/ml imipenem agar with a frequency of $1x10^{-6}$ (4-fold higher than the imipenem MIC). As previously reported, strain BR7 grew on 32ug/ml imipenem agar with a frequency of $2x10^{-7}$ and on 16ug/ml imipenem agar with a frequency of $3x10^{-6}$ (16- and 8-fold higher than the imipenem MIC, respectively) (1).

Mechanism of inoculum effect differs in heteroresistant strains. As previously shown, all heteroresistant KPC-producing strains demonstrated an inoculum-dependent increase in imipenem MICs, while their population profiles showed that the frequency of the expression of imipenem heteroresistance was mediated by a small number of cells, ranging from $2x10^{-7}$ to $3x10^{-6}$ (1). Likewise, our time kill analysis showed that a minimum inoculum of $3.3x10^{6}$ cfu/ml was typically required to mediate population recovery in lethal doses of imipenem. We also showed that the frequency increased for 2 h imipenem-exposed survivors ($1\text{-}2x10^{-6}$), demonstrating again that these survivors were physiologically different from their unexposed counterparts.

We then tested wild-type BR7 and a drug-susceptible *K. pneumoniae* control strain, SF519, to assess whether inoculum-dependent imipenem MICs were mediated by presumptively specialized cells or was a function of the density of cells in each sample (Table 17). We reasoned that if specialized cells such as persisters existed in small numbers in the original inoculum, we should be able to find them if we dispersed the entire inocula into drug wells, and that the cells would mediate growth in lethal doses of imipenem independently of the absolute density of the inocula in each well. We estimated the frequency of heteroresistance under the assumption that a minimum of 1 cell mediated recovery of the population for that well. These frequencies were then derived from the number of positive test wells divided by the total inoculum in the 1ml starting culture. For an inoculum of $4x10^{7}$ cfu/ml, the minimum number of positive samples should have been similar for each of the test groups. In fact, this is what we found for BR7. Positive test wells in each group with a total dispersed inoculum of $4x10^{7}$cfu approximated a minimum frequency of $4\text{-}6x10^{-7}$ irrespective of whether the cell density in each well was $4x10^{7}$ or $4x10^{5}$. This differs from the results we observed with drug-susceptible *K. pneumonia* SF519 which grew at 4-fold over its MIC only in the highest density wells. The estimated frequency for BR7 in this test was similar to our results for imipenem agar-based population analysis (1).

DISCUSSION

We previously identified distinct characteristics that mediate expression of imipenem heteroresistant KPC-producing *K. pneumoniae* strains (1). We demonstrated a correlation between ultimate survival in lethal doses of imipenem to inoculum size, carriage of bla_{KPC} and loss of the outer membrane porin OmpK36. In this study, we strengthened the connection between bacterial physiology and OmpK36 production, showing that the cells that mediated the heteroresistance phenotype – the survivors of the lethal dose of imipenem that killed > 99% of the population - had a different physiology than their unexposed counterparts. In the wild-type heteroresistant strains, expression of imipenem heteroresistance was maximized in conditions of rich media with peptides as a nutritional source. Alternately, in conditions of low osmolarity and lower nutrients, wild-type strain BR7 was still able to express heteroresistance with reduced frequency, and was able to abolish OmpK36 in the presence of imipenem. Addition of glucose under these conditions enhanced heteroresistance. Conversely, expression of heteroresistance was suppressed under conditions of mid-level osmolarity lacking peptides, with only glucose as a

carbon source, and OmpK36 continued to be produced. This observation was similar for wild-type, non-reverter strain BR21.

The BR7 mutants, A1.2, E7 and B3.2, had suppressed heteroresistance in rich media. However, expression was enhanced in low osmolarity NB supplemented with glucose. Like wild-type BR7, these strains could abolish production of OmpK36 in these conditions, but could not do so in minimal media with glucose as a sole carbon source. The mutant strains (A1.2, B3.1, B3.2, E7, E11) fared worse in conditions of high osmolarity and low peptide nutrients, which induced total imipenem susceptibility. This observation is in agreement with a mutation in amino acid transport in strains A1.2 and B3.1. We are conducting further experimentation with mutant B3.2 to understand how mutation in a phage tail protein leads to abolished expression of heteroresistance.

Generation times were different during growth in varied media, as expected. For wild-type BR21, the number of survivors after 2 h of exposure to imipenem in rich media, or in minimal media with glucose was similar, yet the samples grown in glucose-rich media were unable to recover unless the concentration of imipenem was substantially reduced. For the imipenem susceptible mutant B3.1, lag time in the first 2 h of drug-free growth was extended (194–300 minute generation time vs 45-53 minutes for wild-type). B3.1 was also found to have lost the bla_{KPC} gene, which accounts for its increased susceptibility. However, B3.1 and mutant E7 had very slow growth in rich and low nutrient, high-osmolarity media with imipenem, taking more than 3 hours longer to reach mid-logarithmic growth than A1.2 or wild-type BR7 (data not shown). Although additional experiments need to be performed for a more rigorous statistical comparison of growth rates, taken together, the preliminary results suggest that while slow growth favors persistence and drug tolerance, there may be an optimal level of growth required to mediate the porin regulation that seems to be at the core of the expression of heteroresistance.

The link between bacterial metabolism and heteroresistance is also strengthened with the observation that the pronounced inoculum effect we have observed in heteroresistant KPC-producing *K. pneumoniae* strains is mediated by the selection of seemingly specialized cells that survive the initial lethal dose of imipenem. We showed that the size of the starting inoculum is important because these types of cells, which have been termed persisters, are present in stationary phase cultures in very small numbers (6-9). Our experiments argue against starting inoculum-mediated recovery as a function of bacterial density.

Keren et al. observed that persister formation increases during late exponential growth phase, reaching a maximum in stationary phase (10). Early exponential growth dilutes the number of persisters, presumably because they are resuming normal growth. In our previous study we observed a similar range of survivors after 2 and 8 h of imipenem exposure under logarithmic growth conditions compared to stationary phase growth experiments with higher inocula ($p>0.05$, unpublished data, not shown). However, unlike the stationary phase samples, only four in 12 samples (33%) tested under logarithmic growth conditions achieved population recovery. This was compared to near full recovery (76%) observed for samples with similar inocula levels tested under stationary phase conditions ($p=0.01$).

In fact, many of our experimental observations in this and our previous studies show strong similarities between the survivors after 2 h of imipenem exposure and the distinct characteristics of persister cells. To summarize these, a bactericidal dose of imipenem led to the survival of a minor subpopulation (0.1% or less) for all heteroresistant strains tested (1). Population analysis found that a small subset of these initial survivors mediated recovery of the population. Time kill analysis showed that the number of survivors was similar at 2 hours after imipenem exposure among all strains tested, regardless of imipenem susceptibility status, starting inocula, growth phase of the starting cultures, or carriage of bla_{KPC}. Survivors at this point lacked heritable resistance to imipenem as evidenced by an unchanged MIC relative to the unexposed parental strain. No imipenem degradation was observed in the culture supernatant at this point. Together, these observations are consistent with the characteristics of persisters, originally described by Joseph Bigger in 1944, with further characterization by others including their phenotypic tolerance to many classes of antimicrobial drugs (6-12).

Our Group 1-type transposon mutants (A4.2, B1.2, B3.2, E7, B3.1, A1.2) had substantially reduced expression of heteroresistance. Time kill analysis has not yet been performed with these strains, but it is evident that the mutation in the ABC-type amino acid transport cluster is important in some manner, contributing to the ability of the wild-type imipenem survivors to mediate population recovery after losing more than 99% of the initial population. On the other hand, mutants in our Group 2-type cluster have expression of heteroresistance under standard inoculum conditions, which is not observed in any of our wild-type heteroresistant strains studied so far. Our laboratory plans to conduct further experiments with these mutants to explore this difference.

Although we focused this study primarily on the role of bacterial metabolism in expression of imipenem heteroresistance, we observed apparent glucose-mediated killing of wild-type BR21 in the presence of gentamicin, accomplished with 2 h pretreatment with imipenem. This effect was only observed in the pre-enriched cultures that were subsequently exposed to gentamicin in minimal media with glucose, and not in those subsequently exposed to gentamicin in rich media. Unlike what we observed with lowered imipenem MICs in minimal media with glucose, the gentamicin MIC was unchanged in conditions of rich and minimal media. These experiments were based on results produced by Allison et al., who found that metabolites such as glucose and mannitol potentiated the killing of *E. coli* persisters (5). They further demonstrated the clinical relevance of their work in a mouse model of urinary tract infection. Intravenous treatment with mannitol was found to reduce the viability of biofilm formation on catheters, and reduced the spread of infection. Although our heteroresistant KPC-producing *K. pneumoniae* strains are susceptible to gentamicin, evidence indicates they are also susceptible to metabolite-mediated killing and have specific nutritional requirements to fully express heteroresistance. This highlights potential therapeutic avenues of exploration for these strains that pose an urgent and increasing threat to public health.

CONCLUSIONS AND FUTURE DIRECTIONS

Our experiments with wild-type imipenem heteroresistant KPC-producing strains, and the mutants that lost this phenotype, grown in media with varied osmolarity and nutrient content, revealed key differences in expression of heteroresistance and OmpK36 production. With these findings, we are developing a theoretical model to explain the physiologically-induced regulation of *ompK36* expression leading to high-level imipenem resistance. Experiments are now underway in our laboratory to test the coordination of mechanisms described here (Figure 7).

As discussed in Chapter 2, regulation of *ompK35* and *ompK36* expression occurs primarily through the transcriptional control of the EnvZ-OmpR two component system (13-16). In the typical state of our heteroresistant KPC-producing strains grown in high-osmolarity, nutrient-rich Mueller Hinton broth (with no added stressors such as imipenem), EnvZ kinase activity is favored leading to high amounts of phosphorylated OmpR (OmpR~P) in the cell, so that all F-type (located in *ompK35* promoter region) and C-type (in *ompK36* promoter region) binding sites are filled (13, 14, 16-23). Transcription of *ompK35* is thus repressed and *ompK36* expression is activated. *micF* expression is probably a minor factor as it is overshadowed by the repressive effect of OmpR (20). The study strains were found to contain additional putative F-type binding sites in the region upstream of *ompK35* created by the insertion of IS*Ecp1* and *orfB*. The 2 putative sites created by IS*Ecp1* are of low affinity according to the analysis of critical binding residues postulated by Yoshida et al. (16). The putative F2-type site created by *orfB* however, contains critical residues for a high affinity site. OmpR~P may bind to these extra sites prior to the C-type sites to attenuate the levels of *ompK36* transcription. However, binding of OmpR~P must be demonstrated in our strains to show that these are legitimate binding sites. Yoshida et al. concluded that it was unknown how cooperative binding occurs between 2 OmpR~P dimers on the adjacent binding sites in a typical structure (16). It has been shown that OmpR~P can bind as a dimer to an F1- or C1- type alone, but the binding to F1-2-3 and C1-2-3 is unclear. Since our putative alternate binding sites are singlets, we need to demonstrate that cooperative binding of OmpR~R with adjacent sites is not a strict requirement.

Imipenem exposure results in cell wall damage, envelope stress, and creation of reactive oxygen species (ROS), which can lead to transcriptional changes affecting porin expression (21, 22, 24-30). We showed that our heteroresistant strains effectively inactivated low levels of imipenem as a function of bla_{KPC} hydrolysis, combined with reduced or absent expression of *ompK35*, but as the imipenem concentration increased, a majority (>99%) of the population was killed. We identified several mechanisms involved in the recovery of the minority of the population, which we believe our model helps explain.

Inoculum effect versus density. We showed that it was not the density of the cultures per se, but rather, cells with a distinct physiology, present in low numbers in starting cultures, and selected by lethal concentrations of imipenem, that coordinate population recovery subsequent to the killing of the majority of the initial cells. These survivors had many of the phenotypic characteristics of persisters (6, 10, 11). We hypothesize that the reduction in metabolic activity stimulates RpoN-dependent induction of amino acid transport to maintain the level of metabolic

activity required for ensuring eventual population recovery (31-33). Cells in this state are more tolerant to antimicrobial assault (6, 10, 11), and autolysis (33). This part of our model is strengthened by the observation that our amino acid transport mutants had much slower growth rates than the wild type heteroresistant strain, lost the heteroresistant phenotype, and did not abolish OmpK36 production under any of the mid- to high-osmolarity conditions we tested.

Imipenem dose effect. Although the heteroresistant KPC-producing strains were initially protected from imipenem-mediated killing at low drug concentrations, additional mechanisms were employed when strains were subjected to higher imipenem concentrations (exceeding 8ug/ml). We observed that strains began to abolish OmpK36 production at this point (we did not perform quantitative expression analysis to determine whether this starts to occur at lower imipenem concentrations). Our model explains this as a result of increased cell wall damage when imipenem concentrations exceeds the cell's ability to inactivate the drug through KPC enzyme hydrolysis and reduced influx through OmpK35. The disruption causes bulges and instability of the outer membrane (34-36). Membrane disruptions are sensed by EnvZ, which increases its phosphatase activity, leading to lower amounts of OmpR~P in the cell (37-40). Cell envelope stress is also sensed by the RcsCBD pathway (28). *rcsB* is also induced by OmpR~P binding which would hypothetically compete with low affinity C-type binding sites in *ompK36* (41-43). This coordination would both increase capsular polysaccharide production, while attenuating production of OmpK36. Interestingly, the *rcsCBD* locus is situated directly upstream of the *ompK36* promoter region in all of the study strains, which may facilitate cross-phosphorylation of RcsB or RcsD by OmpR (14, 42). This fits with our observation of the increased mucoid appearance in OmpK36-deficient cells (data not shown), which Tsai et al. terms "glistening colonies" (24). In their study, the complementation of OmpK36-deficient cells with a wild-type copy of *ompK36* (but not *ompK35*), reversed the mucoid appearance of the cells. Similarly, we observed that the OmpK36-deficient strains that reverted to wild-type OmpK36 production also lost their mucoid appearance after drug-free passage.

Physiologically-influenced OmpK36 production. When the heteroresistant strains and the transposon mutants were grown in high-osmolarity, nutrient-limited conditions, they had a suppressed or absent heteroresistant phenotype. Imipenem MICs were lower and the mutant strains showed no ability to abolish OmpK36 production. The BR7ΔK36 strain was even more susceptible than its wild-type BR7 counterpart. In fact, its phenotype replicated that of the transposon mutants (B3.1, B3.2, E7, A1.2, E11). Our model explains this with the higher osmolarity of the medium in spite of the low level of nutrients. EnvZ phosphorylation would still occur at a high level, which would continue to promote transcription of *ompK36.* OmpR-P binding to the F4 site would still block *ompK35* expression. In addition, MarA- or SoxS-mediated induction of *micF* expression could inhibit translation of any *ompK35* mRNA that might be produced (14, 27). In contrast, the increased heteroresistance seen in low-osmolarity nutrient media can be explained by the lower levels of phosphorylated OmpR present in the cell which would lead to loss of OmpR~P binding to C-type sites and no transcription initiation of *ompK36.*

Our model has a conflict in that the nutrient deficiency should lead to the RpoN-dependent induction of *rcsB* and competitive inhibition of OmpR-P binding to the *ompK36* promoter (31). We hypothesize that the significantly reduced growth rate we observed in the low-nutrient, high-osmolarity media was below the threshold required for efficient ATP levels and protein synthesis to mediate this protective effect. While slow growing cells are more resistant to killing by β-lactam drugs, there is likely a trade-off with a growth rate that allows continued production of critical proteins and sequestration of ribosomes to allow full protein synthesis during the recovery phase (33). Furthermore, low levels of protein synthesis would impact production of the KPC enzyme, which we have shown is one of the necessary components for ultimate recovery from lethal concentrations of imipenem (1).

Conversely, when we grew the wild-type heteroresistant strains in minimal media with glucose, they lost the heteroresistant phenotype without a substantial reduction in growth in the drug-free controls. Our model explains this through the ability of high energy phosphate groups to phosphorylate OmpR in addition to, or in the absence of EnvZ. Glucose is converted to acetyl-CoA, which then produces acetyl-phosphate, a high energy donor (14, 18). Even if membrane damage reduces amounts of EnvZ phosphorylation of OmpR, acetyl-phosphate could compensate, keeping levels of OmpK36 production high. Furthermore, since glucose is an easily assimilated carbon source, no RpoN-dependent activation would occur to counteract the OmpR binding to *ompK36.*

We have not yet performed the necessary number of experiments, nor performed them with a sufficient number of mutants, to draw solid conclusions regarding the applicability of our results to our hypothetical mechanistic model. We have yet to determine the complete contribution of $bla_{KPC,}$ or other elements contained in its Tn*4401* transposon structure, to phenotypic imipenem heteroresistance. We have not yet explored the contribution of alternate porins such as LamB, which we have detected in outer membrane extracts, and has been reported to occur in conjunction with the loss of OmpK36 (44). Nevertheless, our experiments to date provide preliminary evidence in support of our model. The data from this study clarified the types of experiments we need to undertake to close the gaps in our understanding of the coordination between bacterial physiology and the complex network involved in porin regulation that seems to be at the heart of the imipenem heteroresistance phenotype.

REFERENCES

1. **Adams-Sapper S, Nolen S, Donzelli GF, Lal M, Chen K, Justo da Silva LH, Moreira BM, Riley LW.** 2015. Rapid induction of high-level carbapenem resistance in heteroresistant KPC-producing Klebsiella pneumoniae. Antimicrob Agents Chemother doi:10.1128/AAC.05100-14.
2. **Carlone GM, Thomas ML, Rumschlag HS, Sottnek FO.** 1986. Rapid microprocedure for isolating detergent-insoluble outer membrane proteins from Haemophilus species. J Clin Microbiol **24:**330-332.
3. **Saier MH, Jr., Reddy VS, Tamang DG, Vastermark A.** 2014. The transporter classification database. Nucleic Acids Res **42:**D251-258.
4. **Marchler-Bauer A, Derbyshire MK, Gonzales NR, Lu S, Chitsaz F, Geer LY, Geer RC, He J, Gwadz M, Hurwitz DI, Lanczycki CJ, Lu F, Marchler GH, Song JS, Thanki N, Wang Z, Yamashita RA, Zhang D, Zheng C, Bryant SH.** 2015. CDD: NCBI's conserved domain database. Nucleic Acids Res **43:**D222-226.
5. **Allison KR, Brynildsen MP, Collins JJ.** 2011. Metabolite-enabled eradication of bacterial persisters by aminoglycosides. Nature **473:**216-220.
6. **Shah D, Zhang Z, Khodursky A, Kaldalu N, Kurg K, Lewis K.** 2006. Persisters: a distinct physiological state of E. coli. BMC Microbiol **6:**53.
7. **Lewis K.** 2010. Persister cells. Annu Rev Microbiol **64:**357-372.
8. **Lewis K.** 2007. Persister cells, dormancy and infectious disease. Nat Rev Microbiol **5:**48-56.
9. **Gerdes K, Maisonneuve E.** 2012. Bacterial persistence and toxin-antitoxin loci. Annu Rev Microbiol **66:**103-123.
10. **Keren I, Kaldalu N, Spoering A, Wang Y, Lewis K.** 2004. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett **230:**13-18.
11. **Bigger JW.** 1944. Treatment of Staphylococcal infections with penicillin by intermittent sterilisation. The Lancet **2:**4.
12. **Lewis K.** 2012. Persister cells: molecular mechanisms related to antibiotic tolerance. Handb Exp Pharmacol doi:10.1007/978-3-642-28951-4_8**:**121-133.
13. **Cai SJ, Inouye M.** 2002. EnvZ-OmpR interaction and osmoregulation in Escherichia coli. J Biol Chem **277:**24155-24161.
14. **Delihas N, Forst S.** 2001. MicF: an antisense RNA gene involved in response of Escherichia coli to global stress factors. J Mol Biol **313:**1-12.
15. **Mizuno T, Mizushima S.** 1986. Characterization by deletion and localized mutagenesis in vitro of the promoter region of the Escherichia coli ompC gene and importance of the upstream DNA domain in positive regulation by the OmpR protein. J Bacteriol **168:**86-95.
16. **Yoshida T, Qin L, Egger LA, Inouye M.** 2006. Transcription regulation of ompF and ompC by a single transcription factor, OmpR. J Biol Chem **281:**17114-17123.
17. **Liu X, Ferenci T.** 1998. Regulation of porin-mediated outer membrane permeability by nutrient limitation in Escherichia coli. J Bacteriol **180:**3917-3922.
18. **Liu X, Ferenci T.** 2001. An analysis of multifactorial influences on the transcriptional control of ompF and ompC porin expression under nutrient limitation. Microbiology **147:**2981-2989.

19. **Ramani N, Hedeshian M, Freundlich M.** 1994. micF antisense RNA has a major role in osmoregulation of OmpF in Escherichia coli. J Bacteriol **176:**5005-5010.
20. **Pratt LA, Hsing W, Gibson KE, Silhavy TJ.** 1996. From acids to osmZ: multiple factors influence synthesis of the OmpF and OmpC porins in Escherichia coli. Mol Microbiol **20:**911-917.
21. **Huang L, Tsui P, Freundlich M.** 1992. Positive and negative control of ompB transcription in Escherichia coli by cyclic AMP and the cyclic AMP receptor protein. J Bacteriol **174:**664-670.
22. **Tsui P, Huang L, Freundlich M.** 1991. Integration host factor binds specifically to multiple sites in the ompB promoter of Escherichia coli and inhibits transcription. J Bacteriol **173:**5800-5807.
23. **Inokuchi K, Furukawa H, Nakamura K, Mizushima S.** 1984. Characterization by deletion mutagenesis in vitro of the promoter region of ompF, a positively regulated gene of Escherichia coli. J Mol Biol **178:**653-668.
24. **Tsai YK, Fung CP, Lin JC, Chen JH, Chang FY, Chen TL, Siu LK.** 2011. Klebsiella pneumoniae outer membrane porins OmpK35 and OmpK36 play roles in both antimicrobial resistance and virulence. Antimicrob Agents Chemother **55:**1485-1493.
25. **Kohanski MA, Dwyer DJ, Collins JJ.** 2010. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol **8:**423-435.
26. **Barbosa TM, Levy SB.** 2000. Differential expression of over 60 chromosomal genes in Escherichia coli by constitutive expression of MarA. J Bacteriol **182:**3467-3474.
27. **Poole K.** 2012. Stress responses as determinants of antimicrobial resistance in Gram-negative bacteria. Trends Microbiol **20:**227-234.
28. **Peterson CN, Carabetta VJ, Chowdhury T, Silhavy TJ.** 2006. LrhA regulates rpoS translation in response to the Rcs phosphorelay system in Escherichia coli. J Bacteriol **188:**3175-3181.
29. **Suzuki T, Ueguchi C, Mizuno T.** 1996. H-NS regulates OmpF expression through micF antisense RNA in Escherichia coli. J Bacteriol **178:**3650-3653.
30. **Scott RE, Dousa TP.** 1980. Differences in the cyclic AMP-dependent phosphorylation of plasma membrane proteins of differentiated and undifferentiated L6 myogenic cells. Differentiation **16:**135-140.
31. **Kustu S, Santero E, Keener J, Popham D, Weiss D.** 1989. Expression of sigma 54 (ntrA)-dependent genes is probably united by a common mechanism. Microbiol Rev **53:**367-376.
32. **Kohler T, Harayama S, Ramos JL, Timmis KN.** 1989. Involvement of Pseudomonas putida RpoN sigma factor in regulation of various metabolic functions. J Bacteriol **171:**4326-4333.
33. **Siegele DA, Kolter R.** 1992. Life after log. J Bacteriol **174:**345-348.
34. **Fung-Tomc JC, Huczko E, Banville J, Menard M, Kolek B, Gradelski E, Kessler RE, Bonner DP.** 1995. Structure-activity relationships of carbapenems that determine their dependence on porin protein D2 for activity against Pseudomonas aeruginosa. Antimicrob Agents Chemother **39:**394-399.
35. **Bernabeu-Wittel M, Garcia-Curiel A, Pichardo C, Pachon-Ibanez ME, Jimenez-Mejias ME, Pachon J.** 2004. Morphological changes induced by imipenem and meropenem at sub-inhibitory concentrations in Acinetobacter baumannii. Clin Microbiol Infect **10:**931-934.

36. **Daly KE, Huang KC, Wingreen NS, Mukhopadhyay R.** 2011. Mechanics of membrane bulging during cell-wall disruption in gram-negative bacteria. Phys Rev E Stat Nonlin Soft Matter Phys **83:**041922.
37. **Brown MR, Collier PJ, Gilbert P.** 1990. Influence of growth rate on susceptibility to antimicrobial agents: modification of the cell envelope and batch and continuous culture studies. Antimicrob Agents Chemother **34:**1623-1628.
38. **Srividhya KV, Krishnaswamy S.** 2004. A simulation model of Escherichia coli osmoregulatory switch using E-CELL system. BMC Microbiol **4:**44.
39. **Waukau J, Forst S.** 1999. Identification of a conserved N-terminal sequence involved in transmembrane signal transduction in EnvZ. J Bacteriol **181:**5534-5538.
40. **Inoue K, Matsuzaki H, Matsumoto K, Shibuya I.** 1997. Unbalanced membrane phospholipid compositions affect transcriptional expression of certain regulatory genes in Escherichia coli. J Bacteriol **179:**2872-2878.
41. **Detweiler CS, Monack DM, Brodsky IE, Mathew H, Falkow S.** 2003. virK, somA and rcsC are important for systemic Salmonella enterica serovar Typhimurium infection and cationic peptide resistance. Mol Microbiol **48:**385-400.
42. **Majdalani N, Gottesman S.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu Rev Microbiol **59:**379-405.
43. **Stout V, Gottesman S.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in Escherichia coli. J Bacteriol **172:**659-669.
44. **Garcia-Sureda L, Juan C, Domenech-Sanchez A, Alberti S.** 2011. Role of Klebsiella pneumoniae LamB Porin in antimicrobial resistance. Antimicrob Agents Chemother **55:**1803-1805.

Table 11. Transposon mutants of *K. pneumoniae* BR7 included in this study.[1]

IPM, highest concentration of growth (ug/ml)				
Inocula, cfu/ml	10^8	10^7	10^6	$5x10^5$ (CLSI std)
BR7, wild type	>32	32	4-8	1
BR7ΔK36[2]	>32	>32	>32	>32
BR21, wild type	>32	32	4-8	1
BR21ΔK36[2]	>32	>32	>32	>32
A1.2	>32	16-32	1	<0.5
B3.1	1	0.5	0.25	0.25
B3.2	16	8	2	1
E7	>32	4-8	1	0.25
E11	>32	8-16	2	0.5

[1] Not all BR7 transposon mutants described in this study are represented above. Additional data for imipenem (IPM) heteroresistance at 10^6 and $5x10^5$ cfu/ml inocula for the mutants described in this study are shown in Figure 6.

[2] BR7ΔK36 and BR21ΔK36 are OmpK36-deficient strains derived from exposure of their wild-type counterparts to lethal concentrations of IPM. BR21ΔK36 retains its high level of IPM resistance due to a mutation in *ompK36*, while BR7ΔK36 resumes production of OmpK36 in absence of continued IPM exposure.

Table 12. Identification of the transposon site of insertion in mutants of *K. pneumoniae* BR7.

Mutant strain	Tn5 insertion site[1]	nt accession no.[2]	Domain hits[3]	Accession no.	e-value
	Amino acid ABC transporter cluster				
A1.2, A2.2, A3.2, A4.2, B3.1, B1.2, A5 B2.2, B4.2	ABC-type polar amino acid transport & metabolism,	CP006738	ABC_HisP_GlnQ,	cd03262	3.99e-134,
	ATPase component		GlnQ	COG1126	4.53e-160
	ABC-type amino acid transport, permease component	CP006738	HisM,	COG0765	2.77e-79
	Transmembrane subunit		TM-PBP2	cd06261	5.76e-22
	Glutamine binding domain		PBP2_GlnP	cd13619	2.77e-79
	ABC-type amino acid transport, substrate binding component	CP006738	PBP2_GlnP	cd13619	7.52e-127
	Glutamine binding domain		TM-PBP2	cd06261	5.03e-34
	Extracellular solute binding protein		SBP_bac_3	Pfam00497	1.07e-61
	Permease component		HisM	COG0765	5.29e-89
B3.2, D8	Phage tail, tape measure protein	CP010392	lambda phage tail protein, GenBank accession no. AJB76539	cd276274	1962

[1]Transposon insertion site for mutant strains E7, E11, B4.1 to be determined.

[2] GenBank accession number for top nucleotide alignment.

[3] Domain hits from NCBI conserved domain database and Transporter Classification Database.

Table 13. Effect of growth in minimal media with glucose on survival in lethal imipenem concentrations vs. gentamicin for *K. pneumoniae* BR21.

	2h IPM pretreatment[1]	No pretreatment
Resuspended in:[2]	n (%)	n (%)
M9 glc + IPM	0/21 (0)	1/18 (0.6)
MH +IPM	10/12 (83)	12/15 (80)
M9 glc + gnt	0/12 (0)	11/12 (92)
MH + gnt	11/12 (92)	12/12 (100)
M9 glc only	21/21(100)	6/6 (100)
MH only	6/6 (100)	6/6 (100)

[1] See text for detailed method. In brief, a starting inoculum was treated for 2 h in MH plus a lethal dose of IPM (16ug/ml), washed, and resuspended with media and drugs listed; n indicates how many cultures were positive for growth at 20 h; samples were plated to ensure absence of colony forming units.

[2] M9, minimal salts media; MH, Mueller-Hinton broth; glc, 10mm glucose; gnt, 0.5ug/ml gentamicin; IPM, 16ug/ml imipenem

Table 14. Imipenem and gentamicin MIC for imipenem-exposed *K. pneumoniae* BR21 in varied media.

MIC results for test media used (ug/ml)[1]								
Sample[2]	MH+IPM		M9 glc + IPM		MH + gnt		M9 glc + gnt	
Inoc[3]	10^5	10^6	10^5	10^6	10^5	10^6	10^5	10^6
UN	2	8-16	0.5-1	1-2	0.25	0.25	0.25	0.25
2h	2	8	0.5	2	0.25	0.25	0.25	0.25
8hΔK36	>32	>32	16	>32	0.25	0.25	0.25	0.25
8hK36+	2	8	1-2	1-2	0.25	0.25	0.25	0.25

[1] MH, Mueller-Hinton broth; M9 glc, minimal salts media + 10mm glucose; IPM, imipenem; gnt, gentamicin.

[2] BR21 samples: UN, no IPM exposure; 2h, MIC after 2h of IPM exposure; 8hΔK36, BR21 OmpK36-deficient strain after 8h IPM exposure; 8hK36+, BR21 OmpK36-sufficient strain after 8h IPM exposure. Note that results for heteroresistant K. pneumoniae BR7 are the same, except the IPM MIC for the BR7 8hΔK36 in M9 glc is the same as for the BR7 unexposed sample (Table 15).

[3] Inoc: starting inoculum of MIC test, cfu/ml; 10^5 cfu/ml represents the CLSI reference standard;

Table 15. Expression of heteroresistance due to growth in media with varied nutrient and osmolarity composition.

				Growth in IPM (ug/ml), 10^6 cfu/ml inocula [1]						Growth relative to MH[2]
Strain	Media[3]	Drug-free[4]	Omp K36[5]	0.5	1	2	4	8	16	
BR7	MH	.79	-	*	*	*	*	H	H	-
	6% MH	.24	-	*	H					↓
	M9 glc	.58	+	*	*	H				↓
	NB	.56	-	H	H	H				↓
	NB glc	.70	-	*	H		H	H		~
BR7ΔK36	MH	.76	-	*	*	*	*	*	>	-
	6% MH	.23	ND	*						↓
	M9 glc	.48	+	*	*					↓
	NB	.68	ND	*	H		H	H		↓
	NB glc	.47	-	*	H					↓
BR21	MH	.75	+	*	*	*	*	H	H	-
	6% MH	.28	ND	*	*	H				↓
	M9 glc	.50	+	*	*					↓
	NB	.54	ND	H						↓
	NB glc	.40	ND	*				H		↓
BR21ΔK36	MH	.70	-	*	*	*	*	*	>	-
	6% MH	.21	ND	*	*	*	*	*	>	=
	M9 glc	.57	-	*	*	*	*	*	>	=
	NB	.40	ND	*	*	*	*	*	>	=
	NB glc	.47	ND	*	*	*	*	*	>	=
B3.2 mut	MH	.98	+	*	*	*				-
	6% MH	.22	+	*						↓
	M9 glc	.57	+	*	*					↓
	NB	.55	-	*	H					↓
	NB glc	.61	-	*	*	*	H			↑
E11 mut	MH	.72	+	*	*	*	H	H		-
	6% MH	.25	ND	*	H	H				↓
	M9 glc	.51	+	*	*	H				↓
	NB	.63	-	H						↓
	NB glc	.47	-	H		H	H			~
E7 mut	MH	.74	+	*	*	H				-
	6% MH	.17	ND							↓
	M9 glc	.65	+	*	*	H				=
	NB	.44	ND	H						↓
	NB glc	.55	+	*	H					↓
A1.2 mut	MH	.87	+	*	*	*				-
	6% MH	.24	+							↓
	M9 glc	.56	+	*	*	H				=

	NB	.53	-	*	H	H	H			↑
	NB glc	.61	-	*	H		H	H		↑

[1] *, homogeneous resistance, all sample wells growth positive; H, heterogeneous growth, 1-2 sample wells positive per replicate or per experiment; dark grey shaded boxes represent concentrations with no growth that fall between a lower and higher concentration with growth, or no growth at the lowest concentration of the test (0.25ug/ml); >, growth ≥32ug/ml.

[2] Increase (↑), decrease (↓), similar (~), or equivalent, (=), growth in imipenem in varied media types relative to growth in MH.

[3] Media used in imipenem (IPM) MIC test: MH, Mueller-Hinton, cation-adjusted, 300mOsm/L (high osmolarity); 6%, 6% MH, 94% M9 minimal salts media, 200mOsm/L (mid-level osmolarity); M9glc, M9 minimal salts with 10mm glucose, 200mOsm/L (mid-level osmolarity); NB, Nutrient Broth, low osmolarity (≤100mOsm/L); NBglc, NB with 10mm glucose.

[4] Growth at 20 h in drug-free media was measured by OD_{600}.

[5] OmpK36 production in specified media in highest concentration of IPM growth, SDS-PAGE analysis; +, porin present; -, porin absent. ND, not determined. All strains showed the presence of OmpK36 in drug-free growth in each of the media types (data not shown).

Table 16. Effect of growth in rich or minimal media with glucose on generation time and survival for *K. pneumoniae* BR21.

2 h enumeration of IPM-exposed cultures and population recovery							
		MH			M9 + 10mm glucose		
cfu/ml[1]	IPM (ug/ml)	2 h cfu/ml	Gen time (min)	Rec[2]	2 h cfu/ml	Gen time (min)	Rec[2]
$5x10^6$	16	$9x10^3$	--	3/3	$5x10^3$	--	0/3
$5x10^6$	4	$1x10^5$	--	3/3	$8x10^4$	--	1/3
$5x10^6$	0	$2x10^8$	26, 28	-	$1x10^7$	91, 540	-
Growth of 2 h IPM-exposed cultures, resuspended in drug-free media							
		MH			M9 + 10mm glucose		
cfu/ml[3]		2 h cfu/ml	Gen time (min)		2 h cfu/ml	Gen time (min)	
$9.8x10^3$		$2.3x10^4$	97		$7.5x10^3$	↓	
$9.5x10^3$		$2x10^4$	112		$3x10^3$	↓	
$9x10^3$		$2.5x10^4$	81		$3x10^3$	↓	

[1] Starting cfu/ml for imipenem (IPM) exposure.

[2] Number of samples achieving population recovery by 20 h of growth in IPM.

[3] Starting cfu/ml of cultures after 2 h IPM exposure, washing and resuspension in either MH or M9 + glucose

MH, Mueller-Hinton broth, cation-adjusted; M9, minimal salts media with 10mm glucose.

Table 17. Comparison of inoculum effect as a function of cell density vs. frequency of putatively specialized cells.

Strain	Total inoc.[1]	Well inoc (cfu/ml)	n	%	Freq[2]	Exp n[3]
BR7	$4x10^8$	$4x10^7$	10/10	100	$3x10^{-8}$	10
	$4x10^7$	$2x10^6$	16/18	89	$4x10^{-7}$	12
	$4x10^7$	$1x10^6$	16/36	44	$4x10^{-7}$	12
	$4x10^7$	$4x10^5$	23/96	24	$6x10^{-7}$	12
SF519	$7x10^8$	$7x10^7$	9/10	90	$2x10^{-8}$	10
	$7x10^7$	$4x10^6$	0/19	0	0	10
	$7x10^7$	$2x10^6$	0/38	0	0	10
	$7x10^7$	$7x10^5$	0/97	0	0	10

[1] Starting inocula were prepared in 1ml volumes; each sample was dispensed in full into 10, 20, 40, or 100 wells of a 96-well plate with imipenem (BR7, 16ug/ml; SF519, 1ug/ml); n is the number of positive wells of the total wells dispersed after 18-20 h of growth (the total n of wells was less than the target number due to pipetting variations).

[2] Frequency of heteroresistant subpopulation in lethal dose of imipenem (IPM) assumed a minimum of 1 cell/well mediated recovery of the population and was calculated as the number of positive wells divided by the total inoculum in 1ml of starting culture.

[3] Expected frequency used was $3x10^{-7}$ based on previously published data (1).

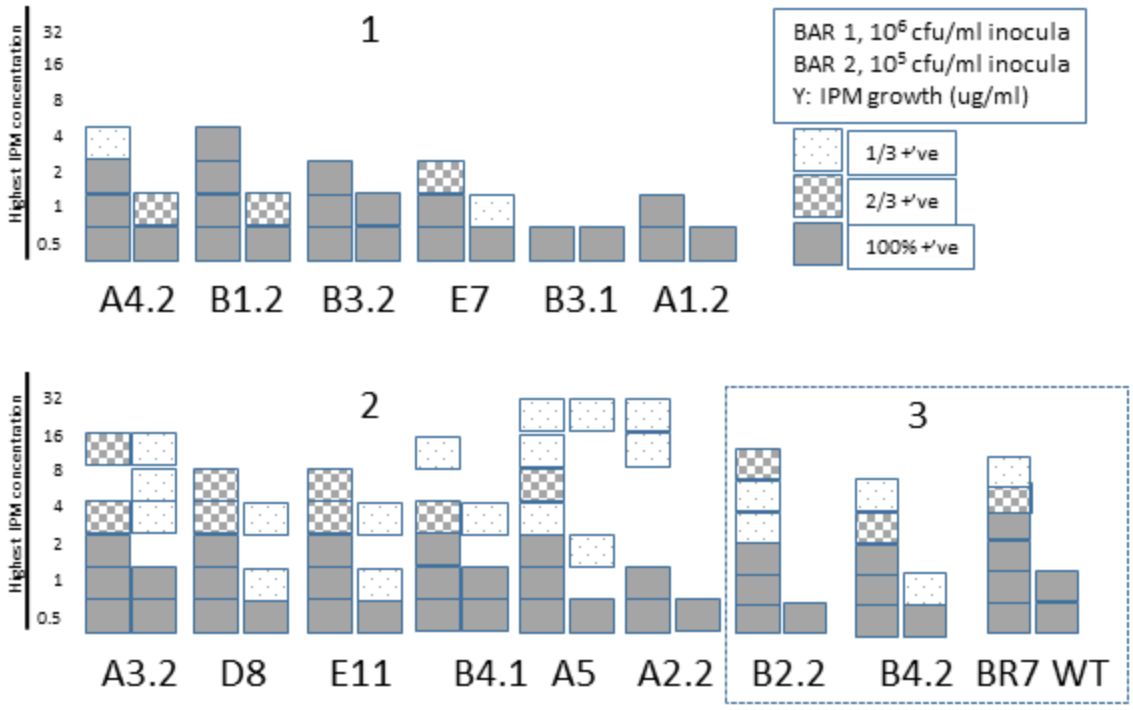

Figure 6. Imipenem (IPM) heteroresistance in transposon mutants of heteroresistant KPC-producing *K. pneumoniae* BR7. Each box represents 3 replicates tested in the imipenem concentration marked on the Y-axis. Differentially shaded boxes represent less than all 3 triplicates growing in that concentration (see boxed legend). Gaps between boxes indicate lack of growth of any of the 3 triplicates in that concentration. Note that these gaps in growth are not seen in BR7 (or any of the other heteroresistant strains we have studied). For each strain, the first column of boxes represents growth in the highest concentration of imipenem for test inocula of $\geq 10^6$ cfu/ml, while the second column is for test inocula of $5x10^5$ cfu/ml (reference standard). Note: the naming convention for the transposon mutants was derived from the initial selection screen, not from their phenotypic group placement (1-3) in this figure.

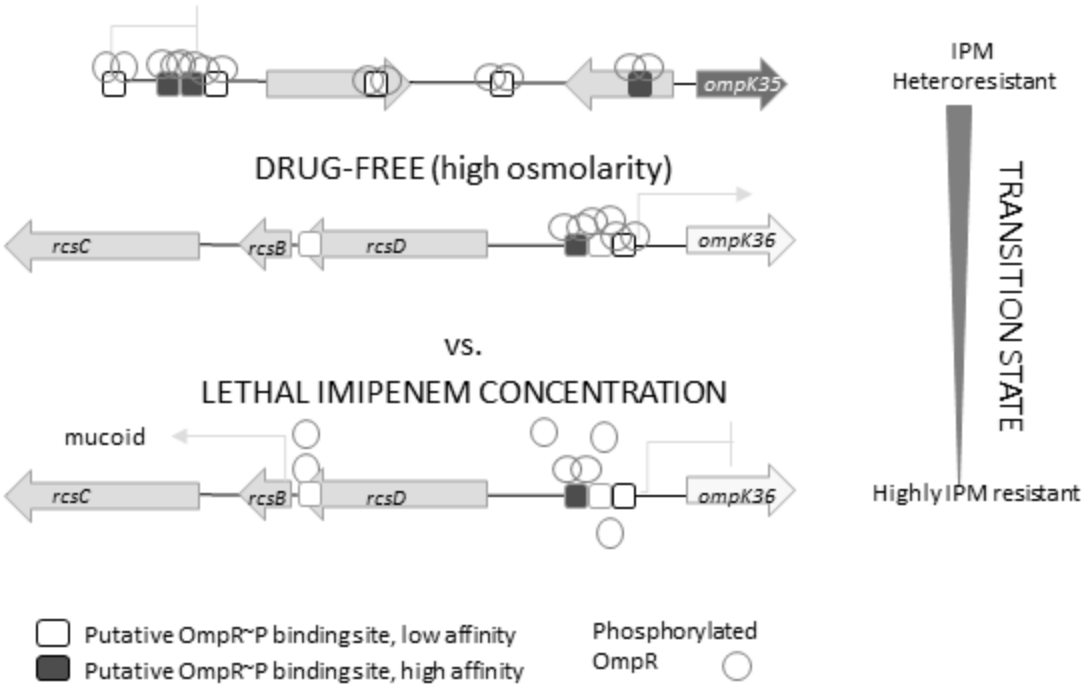

Figure 7. Summary of a hypothetical model of mechanisms mediating the expression of imipenem heteroresistance and the transition to high-level resistance: Heteroresistance is a transition state; as the concentration of carbapenem drug increases, the ability to inhibit cell wall damage through KPC-mediated drug inactivation is exceeded; the majority of the population is killed such that only persisters survive. Recovery depends on sufficient protein synthesis to occur for production of KPC enzyme and other essential proteins for eventual recovery. RcsCBD activation via the membrane damage sensing pathway would compete with C-type sites in the *ompK36* promoter region for OmpR interaction resulting in increased capsule production and decrease in OmpK36 production. For simplicity, not all genes in the *ompK35* and *ompK36* region are shown or fully labeled here, and only the *ompK36* genetic region is shown in the transition from the drug-free conditions to growth in imipenem. Refer to Chapter 2 text and Figures 3 and 4 for the detailed genetic arrangements of *ompK35* and *ompK36*.