Heteroresistance via beta-lactamase gene amplification threatens the beta-lactam pipeline

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ABSTRACT

Novel antibiotics are threatened by rapid emergence of resistance. Heteroresistance (HR) is a form of resistance where a phenotypically unstable and often undetected minority resistant subpopulation co-exists with a susceptible population. We observed HR to cefiderocol, a novel beta-lactam developed to resist beta-lactamases including ESBLs. The resistant subpopulation was a continuum; increasing gene amplification of otherwise ineffective ESBLs mediated increased resistance in decreasing numbers of cells. Surprisingly, ESBL amplification further increased when mutations or beta-lactamase inhibitors (BLI) were employed to inhibit activity, demonstrating that amplification overcomes inefficient activity. After one exposure to sub-breakpoint concentrations of cefiderocol/BLI, this enhanced amplification mediated resistance in an isolate collected before clinical introduction of the drugs. Therefore, amplification allows HR isolates to employ pre-existing, otherwise ineffective beta-lactamases to overcome novel beta-lactam/BLIs without new, stable evolution. This phenotypic flexibility is undetected during drug development and reveals an unappreciated threat to beta-lactam/BLIs that dominate the antibiotic pipeline.

INTRODUCTION

Antibiotic resistance is a growing crisis causing over 1 million worldwide deaths in 2019[1]. Without significant intervention, this number is predicted to rise to 10 million annual deaths by 2050[2]. The staggering death toll can be partly attributed to the widespread dissemination of antibiotic resistance, the rapid emergence of resistance to novel antibiotics, and a lagging pipeline for the development of novel classes of antibiotics. Variations of beta-lactams and beta-lactamase inhibitors have dominated the antibiotic pipeline in part because of their efficacy, low toxicity, and the many obstacles[3] that hinder the development of completely novel antibiotic classes. In 2020, 47% of outpatient antibiotic prescriptions in the United States were beta-lactams, nearing 100 million[4]. Five of nine new antibiotics expected to treat highly resistant Gram-negative pathogens and approved from 2014 to 2020 were beta-lactams, and as of 2020, 7/7 antibiotics in Phase III testing were beta-lactams[5]. This reliance on beta-lactam antibiotics is a healthcare vulnerability since resistance emerges quickly following clinical introduction, although the reasons for this rapid resistance are unclear[6].

Cefiderocol is a recently FDA-approved beta-lactam consisting of a siderophore linked to a novel hybrid cephalosporin. The siderophore portion facilitates the transport of cefiderocol into the bacterial cell via siderophore receptors, while the late generation cephalosporin (a subclass of beta-lactams) moiety consisting of parts of ceftazidime and cefepime was designed to circumvent hydrolysis by beta-lactamases which cleave and inactivate beta-lactams. Enzymatic assays with cefiderocol showed relative resistance to hydrolysis by Ambler class A and D (serine) and B (metallo-) carbapenemases[7,8], as well as class C AmpC-beta-lactamases[9]. Broad screens of clinical isolates using conventional antimicrobial susceptibility testing (broth microdilution; BMD) revealed low minimum inhibitory concentrations (MIC) against carbapenemase-, metallo-beta-lactamase-, and extended-spectrum beta-lactamase (ESBL)-producing isolates[10-14], suggesting that cefiderocol would be an efficacious new antibiotic for difficult to treat infections caused by Gram-negative pathogens. However, in a recent clinical trial (CREDIBLE-CR) for the treatment of serious infections caused by carbapenem-resistant isolates, higher than expected rates of cefiderocol treatment failure occurred[15]. In particular, cefiderocol was associated with increased all-cause mortality in patients with infections caused by carbapenem-resistant *Acinetobacter baumannii* (CRAB).

Antibiotic treatment failure can be caused by heteroresistance (HR), a form of antibiotic resistance in which a minority population of resistant cells co-exists with a majority susceptible population (Extended Data Figure 1a). Treatment with a given antibiotic prevents the growth of

the susceptible cells while the resistant subpopulation grows and dominates the population, distinguishing this form of resistance from persistence or tolerance. When the antibiotic is removed, the resistant subpopulation returns to its original homeostatic frequency (Extended Data Figure 1b). We and others have observed HR to all classes of antibiotics tested[16,17] and have shown that HR can cause treatment failure in murine models of infection[16,18,19].

To investigate the discordance between the widespread susceptibility to cefiderocol in laboratory testing and the underwhelming patient outcome in clinical testing, we curated a collection of isolates representative of those in the CREDIBLE-CR trial, and from patients pre-dating the clinical introduction of cefiderocol. We observed a substantial frequency of HR especially among CRAB isolates (>50%), and the rate of HR for each species tested closely matched their all-cause mortality rate in the clinical trial[20,21]. These data suggest that undetected cefiderocol HR may have contributed to treatment failure in the Phase III testing of this antibiotic.

The molecular basis of cefiderocol HR is unknown. In this study, we investigated mechanisms of cefiderocol HR in Gram-negative clinical isolates and found that beta-lactamase gene amplification generates a continuum of subpopulations resistant to cefiderocol, where decreasing numbers of cells have increasing amplification and thus resistance. Genetic inhibition or use of beta-lactamase inhibitors (BLI) to reduce beta-lactamase activity led to enhanced beta-lactamase gene amplification, inversely correlating amplification with beta-lactamase function. Exposure to sub-inhibitory concentrations of cefiderocol/BLI led to increases in ESBL gene amplification sufficient to resist the combination. Therefore, amplification enables cellular subpopulations to exploit otherwise ineffective ESBLs to resist cefiderocol, and additional amplification facilitates adaptation to the addition of BLIs. These data lead to novel insights on the population dynamics of HR and how unstable, phenotypic resistance can be used by bacteria to confront new antibiotic threats, even in the absence of stable evolutionary changes. This phenotypic plasticity is undetected during antibiotic development and is a threat to the antibiotic pipeline, which is dominated by beta-lactams/BLIs.

RESULTS

Amplification of an ESBL generates a cefiderocol resistant subpopulation

We identified cefiderocol HR in an isolate of *Enterobacter cloacae*, strain RS[19], by population analysis profile (PAP) in which the frequency of any resistant cells is quantified by

plating dilutions at an array of drug concentrations (Extended Data Figure 1). The frequency of the RS cefiderocol resistant subpopulation at the clinical breakpoint concentration (which differentiates susceptible and resistant isolates[22]) was 1 in 20,000, in contrast to ESBL89, a susceptible isolate in which the entire population is killed at concentrations below the breakpoint (Figure 1a).

We investigated the dynamics of the RS resistant subpopulation during cefiderocol treatment. The resistant subpopulation replicated in cefiderocol, demonstrating that it is not a population of persister cells, which do not replicate in the presence of antibiotic[23] (Figure 1b). A second hallmark of HR is phenotypic instability of the resistant subpopulation. To test whether the resistant subpopulation was stable or unstable, we grew RS in cefiderocol, and then serially passaged in the absence of the drug. We observed enrichment of the resistant subpopulation in cefiderocol and the subsequent return to the pre-selection frequency in its absence, consistent with unstable heteroresistance[17] (Figure 1c). Together, these data indicate that RS exhibits HR to cefiderocol.

We next set out to determine if the resistant subpopulation was genetically distinct from the majority susceptible population. We sequenced the genome of RS after growth in media with cefiderocol (>99% resistant cells) or without (>99% susceptible cells). Bioinformatic analysis revealed a 9.5 kilobase pair (kbp) region in the chromosome of the resistant cells grown in cefiderocol that was present at an increased frequency of ~15x (which we describe as being “amplified” or exhibiting gene amplification). This region included *blaSHV-5*, encoding a class A extended-spectrum beta-lactamase (ESBL; Figure 1d). We used qPCR to confirm that several genes within the amplified region were present at levels similar to those observed from genome sequencing following growth in cefiderocol (Extended Data Figure 2). As a control, we quantified the frequency of genes flanking the amplicon and detected no change with or without cefiderocol (Extended Data Figure 2). These data correlate amplification of a region including a beta-lactamase with the resistant subpopulation of cells in a cefiderocol HR clinical isolate.

To test whether this amplified region was responsible for cefiderocol HR, we created in-frame unmarked deletions of the entire amplicon region (Δamplicon) or *blaSHV-5* alone (*ΔblaSHV-5*) and tested the strains by PAP. The resistant subpopulation was not present in the Δamplicon mutant nor in the Δ*blaSHV-5* mutant. Complementation by reintroduction of *blaSHV-5* at its native site in the Δ*blaSHV-5* mutant restored the resistant subpopulation (Figure 1e). Thus, *blaSHV-5* is required for the presence of the cefiderocol resistant subpopulation.

Gene amplification can result from a RecA-dependent[24] tandem duplication event dependent on homologous sequences flanking the amplified region. We observed that the 5' and 3' genes flanking the *blaSHV-5* amplicon are co-directional transposases with 100% nucleotide identity which could potentially support homologous recombination. We designed a PCR primer pair that would yield a product only if the amplicon was duplicated in the same orientation, with a product size indicative of only one intervening copy of the transposase. After growth of RS in cefiderocol, we observed a PCR product indicative of the duplication event (Extended Data Figure 3). Importantly, the duplication event was also detected in RS prior to cefiderocol exposure, albeit at a greatly reduced intensity, consistent with a pre-existing, minor subpopulation of cells exhibiting *blaSHV-5* gene amplification. To determine if RecA was required for amplification of the *blaSHV-5* region, we created an in-frame deletion of *recA* and performed PAP. The resistant subpopulation was absent in the Δ*recA* mutant and this phenotype was complemented by *recA* in trans (Figure 1f). These data indicate that RecA is indeed required for cefiderocol HR.

Taken together, these data led to a model where in the absence of cefiderocol, the majority of the cells have a single copy of the *blaSHV-5* amplicon and a minority exhibit RecA-mediated, *blaSHV-5* gene amplification. In contrast, in the presence of cefiderocol, there is enrichment of the cells with *blaSHV-5* gene amplification. To further test this model, we grew RS with or without increasing concentrations of cefiderocol and quantified *blaSHV-5* gene frequency. Interestingly, as the concentration of cefiderocol increased and the frequency of the resistant subpopulation decreased, the average number of *blaSHV-5* copies in the surviving resistant subpopulation increased (Figure 1g). At 8 µg/mL, 1 in 5,000 cells survived with an average gene frequency of 2, whereas at 32 µg/mL, 1 in 150,000 cells survived with an average gene frequency of 20. This suggested that the cells surviving in increasing concentrations of cefiderocol require increasing *blaSHV-5* amplification to withstand the additional stress. Furthermore, these data surprisingly indicate that the cefiderocol resistant subpopulation is actually a continuum of subpopulations with increasing *blaSHV-5* gene frequency and resistance levels (Figure 1h).

Additionally, we selected 15 resistant colonies from agar plates containing cefiderocol, grew each in broth, and then performed qPCR to quantify the number of *blaSHV-5* copies. The extent of *blaSHV-5* gene amplification in the resistant colonies was always greater than 1 but with variable magnitude, consistent with *blaSHV-5* amplificaiton heterogeneity in the population. Thus, among the resistant cells surviving at a given concentration of cefiderocol, the level of

blaSHV-5 amplification is not uniform. For example, at 8 µg/ml cefiderocol, a majority of surviving cells would have an average *blaSHV-5* frequency of 2 (as mentioned above), but minorities of surviving cells would have average *blaSHV-5* frequencies of between 6 and 20 and be capable of also surviving at 16 µg/ml or 32 µg/ml, respectively (Figure 1g and Extended Data Figure 4).

Beta-lactamase amplification in carbapenem-resistant isolates

Having established that an ESBL causes cefiderocol HR in *E. cloacae* RS, we investigated whether beta-lactamase amplification might be a widespread mechanism to generate cefiderocol resistant subpopulations in HR isolates. As a first step, we attempted to determine which HR isolates might require a beta-lactamase to resist cefiderocol. If the resistant subpopulation in an HR isolate was absent when treated with cefiderocol in combination with a beta-lactamase inhibitor (BLI), then a beta-lactamase would be implicated. We performed PAP with cefiderocol alone or with the BLIs clavulanate or avibactam for each of the 27 carbapenem-resistant (CR) Enterobacterales and 65 CR-*Acinetobacter* from our surveillance collection that exhibited cefiderocol HR. Clavulanate had minimal effect for most isolates, consistent with its scope of inhibition being limited to only some members of class A and D beta-lactamases[25]. Avibactam, which inhibits class A, C, and some class D serine beta-lactamases, but not class B metallo-beta-lactamases, was effective at eliminating the resistant subpopulation in a majority of isolates of each group, implicating serine beta-lactamases as being required for cefiderocol HR in these isolates (Extended Data Figure 5).

In order to further investigate the role of beta-lactamases in cefiderocol HR, we selected representative isolates of multiple species; CR-*K. pneumoniae* isolate Mu1413 and CR-*Acinetobacter* isolates Mu1956 and Mu1984. We next assessed whether the resistant subpopulation in these strains was unstable by enriching it in cefiderocol and then passaging the strains into drug-free media. The resistant cells of each strain were enriched in cefiderocol and returned towards the baseline frequency subsequently in the absence of the drug, demonstrating the unstable HR phenotype in all three isolates (Extended Data Figure 6). To determine if the resistant subpopulation in each strain harbored beta-lactamase amplifications, we sequenced each isolate after growth with or without cefiderocol. When grown with cefiderocol, Mu1413 demonstrated amplification of a 4.7 kbp region that contained *blaSHV-28*, encoding a distinct class A ESBL (Figure 2a). The cefiderocol resistant subpopulation was eliminated by the addition of avibactam, consistent with the susceptibility of SHV-28 to avibactam[26] (Figure 2b). In cefiderocol, Mu1956 exhibited amplification of a large 55.2 kbp

region that included the *blaADC-30* gene, encoding an ESBL which is one of the most prevalent *Acinetobacter*-derived cephalosporinase (ADC, class C) variants[27] (Figure 2c). The resistant subpopulation in Mu1956 was also eliminated by addition of avibactam (Figure 2d). An amplicon with similar architecture including an ESBL was identified in Mu1984, with the *blaADC-33* gene encoding a related ADC[28]; this resistant subpopulation was also eliminated by avibactam (Figure 2e-f), consistent with the susceptibility of ADCs to avibactam[27]. Together, these data indicate that cefiderocol HR isolates can harbor resistant subpopulations with ESBL gene amplifications.

Beta-lactamase amplification overcomes addition of beta-lactamase inhibitors

We observed that the magnitude of gene amplification in the resistant subpopulation correlates with cefiderocol concentration (Figure 1g). This suggested that strains require more beta-lactamase function to resist higher concentrations of cefiderocol. As BLIs reduce the activity of beta-lactamases, we therefore hypothesized that if a strain were to survive in a given concentration of cefiderocol plus BLI, it would require more beta-lactamase to survive than in the same concentration of cefiderocol alone. The resistant subpopulation of RS was eliminated by clavulanate, a known BLI with activity against SHV-5 (Figure 3a). We therefore selected resistant colonies of RS on 8 µg/mL cefiderocol and increasing concentrations of clavulanate (Figure 3b). As the frequency of the resistant subpopulation decreased with increasing clavulanate, we observed a concomitant increase in *blaSHV-5* amplification. This suggests that with the concentration of cefiderocol held constant, cells required a dose-dependent increase in *blaSHV-5* to survive the increasing clavulanate concentration. This result, therefore, inversely links *blaSHV-5* amplification and SHV-5 function. To quantify the magnitude of *blaSHV-5* amplification in cells grown on cefiderocol/clavulanate by an alternative assay, we performed droplet digital PCR and observed a similar extent of *blaSHV-5* amplification (Extended Data Figure 7). These data are consistent with our hypothesis that the extent of gene amplification correlates with the magnitude of beta-lactamase function required for the bacteria to survive.

We next tested whether this phenomenon occurs in the carbapenem-resistant, cefiderocol HR isolates that harbored cefiderocol resistant subpopulations with amplified ESBLs (Figure 2). Each strain was grown with or without cefiderocol, as well as in media with cefiderocol and the highest concentration of avibactam in which the strain could survive. Growth in cefiderocol or cefiderocol/avibactam similarly enriched the cefiderocol resistant subpopulation (Figure 3c, 3e, 3g). In *K. pneumoniae* Mu1413, cells that survived in cefiderocol/avibactam had a 4-fold increase in *blaSHV-28* gene levels relative to the cells grown in cefiderocol alone

(Figure 3d). The presence of avibactam increased ESBL gene frequency in both CRAB strains as well, suggesting the increased magnitude of amplification overcomes enzyme inhibition (Figure 3f, 3h).

Magnitude of amplification correlates with inhibition of beta-lactamase activity

Our observation that the addition of BLIs led to increased ESBL gene amplification (Figure 3) is consistent with our hypothesis that in order to survive a given cefiderocol exposure, cells would require more copies of a beta-lactamase if it has lower activity. To directly test this hypothesis, we used a genetic approach, generating point mutants in *blaSHV-5* that are known to have reduced activity. Using strain RS, we created the point mutant M69I, which was previously demonstrated[29] to have reduced activity towards the cephalosporins cephalothin and cefotaxime and also generated the S238A;K240E mutations, converting SHV-5 to the parental, non-extended-spectrum beta-lactamase SHV-1 which has reduced activity towards cephalothin, ceftazidime, and cefotaxime relative to SHV-5[30]. The mutated and original coding sequences were inserted at the Δ*blaSHV-5* locus, creating Δ*blaSHV-5*::*blaSHV-5* M69I (*blaSHV-5* M69I, encoding SHV-5 M69I), Δ*blaSHV-5*::*blaSHV-5* S238A;K240E (*blaSHV-5* S238A;K240E), and the wildtype control Δ*blaSHV-5*::*blaSHV-5* (*blaSHV-5*).

We performed PAP and observed that the strains encoding either SHV-5 M69I or SHV-5 S238A;K240E harbored subpopulations with reduced resistance (only surviving up to 4 µg/ml cefiderocol) as compared to the strain encoding SHV-5 (survives up to at least 32 µg/mL cefiderocol (Figure 4a). However, the strains encoding SHV-5 M69I and SHV-5 S238A;K240E exhibited greater resistance than the Δ*blaSHV-5* mutant which only survived to 2 µg/mL (Figure 4a). These data are consistent with SHV-5 M69I and SHV-5 S238A;K240E having lower activity towards cephalosporins than SHV-5[29,30]. We next quantified the average level of *blaSHV-5* amplification in bacteria grown with or without 4 µg/mL cefiderocol, a concentration at which at least some of the cells of each strain could survive (Figure 4a). We observed increased amplification of genes encoding SHV-5 M69I and SHV-5 S238A;K240E compared to SHV-5 (Figure 4b). Together, these data show that genetic inhibition of SHV-5 activity resulted in a corresponding increase in gene amplification in the surviving cells.

Taken together, we have established that 1] there is a pre-existing continuum of subpopulations with differing levels of *blaSHV-5* amplification and corresponding cefiderocol resistance levels present in RS (Figure 1g) and 2] that the magnitude of amplification inversely correlates with SHV-5 function, which can be inhibited through chemical or genetic means

(Figures 3b and 4b). These data clearly indicate that within RS, the extent of *blaSHV-5* amplification is flexible and not fixed. Therefore, we set out to test whether serial passage of the cefiderocol resistant population could select for cells with increasing *blaSHV-5* gene levels and corresponding resistance. We collected the resistant subpopulation of the strain encoding SHV-5 from plates with 4 µg/mL cefiderocol (Figure 4b) and passaged sequentially to 8 µg/ml and 16 µg/mL cefiderocol, performing PAP and quantifying *blaSHV-5* gene frequency after each passage. Serial selection resulted in subpopulations with amplification levels that increased from an average of 1.3 to 15 (Figure 4c). The frequency of the resistant subpopulations in the strain encoding SHV-5 increased in abundance with each passage in increasing concentrations of cefiderocol, yielding an approximate 3,000-fold increase in the frequency of the subpopulation resistant to 32 µg/mL after the final passage relative to the baseline (Figure 4d). We then quantified the extent of gene amplification from colonies resistant to 32 µg/mL cefiderocol after the final passage and found that it additionally increased to an average of 19 (Figure 4e).

We performed the same experiment with strains encoding SHV-5 M69I and SHV-5 S238A;K240E. We observed a similar increase in gene amplification from baseline following passage (Figure 4f and 4i) and increases in the both the frequency of the subpopulation and the highest concentration of cefiderocol on which the subpopulation survived (Figure 4g and 4j). The abundance of genes encoding SHV-5 M69I and SHV-5 S238A;K240E was greater than SHV-5 at each step, and of note, the extent of gene amplification in the colonies resistant to 32 µg/mL cefiderocol approached an average of nearly 200 for *blaSHV-5* M69I and 42 for *blaSHV-5* S238A;K240E compared to 19 for *blaSHV-5* (Figure 4h, 4k, and 4e), These data confirm that the mutants with reduced activity require enhanced *blaSHV-5* gene amplification to survive, and underscore the potential for vast *blaSHV-5* amplification and resulting increases in cefiderocol resistance.

Amplification enriched by antibiotic exposure overcomes beta-lactamase inhibitors

The observations that serial exposure to cefiderocol selected for an increase in *blaSHV-5* gene amplification and greatly increased the frequency and resistance level of the resistant subpopulations led us to hypothesize that similar exposure might drive sufficient amplification of *blaSHV-5* to overcome inhibition by clavulanate. We showed earlier that the extent of *blaSHV-5* amplification in RS was greater with increasing clavulanate (Figure 3b), but cells only survived up to 8 µg/mL cefiderocol plus 0.125 µg/mL clavulanate. We tested whether serial passage would allow survival of RS to at least 16 µg/mL cefiderocol plus 2 µg/mL clavulanate which are the concentrations that would likely be used clinically; the CLSI breakpoint for cefiderocol is 16

µg/mL and the breakpoint concentration of clavulanate when used in combination with the beta-lactam ticarcillin is 2 µg/mL[22]. We sequentially harvested resistant cells from the highest concentration of cefiderocol/clavulanate on which they survived, and subsequently grew them on higher concentrations (Figure 5). Repeating this process, we observed sequential survival on 8 µg/mL /0.06 µg/mL, 16 µg/mL /0.25 µg/mL, and 8µg/mL/4µg/mL cefiderocol/clavulanate (Figure 5a). When we quantified the extent of amplification of *blaSHV-5* in cells surviving in each of the three aforementioned conditions, we detected corresponding increases from 21 to 40 to 63 (Figure 5a). After growth in each sequential condition, PAP was performed and we observed a stepwise increase in the frequency of the resistant subpopulation (Figure 5b). An additional increase to 87 copies of *blaSHV-5* was observed in cells surviving on 16 µg/mL cefiderocol after the final passage (Figure 5c). This demonstrates a proof of principle that amplification of a beta-lactamase can overcome addition of an otherwise effective BLI to a beta-lactam.

Mu1956 was HR to cefiderocol, but our screen found it susceptible to cefiderocol/avibactam (Figure 2d; Extended Data Figure 5), suggesting the addition of avibactam could increase the utility of cefiderocol against this strain. However, based on our finding that beta-lactamase amplification can overcome the addition of an effective BLI (Figure 3), we investigated whether *blaADC-30* amplification could generate a population of cells resistant to the breakpoint concentrations of cefiderocol/avibactam. We first grew Mu1956 in broth containing 4 µg/mL cefiderocol plus 1 µg/mL avibactam, representing one quarter the breakpoint concentrations of each drug. Exposure to sub-breakpoint concentrations of drug is likely to occur for some cells in the population during infection. After a single exposure to sub-breakpoint cefiderocol/avibactam, *blaADC-30* gene frequency increased in most replicates (Figure 5d) and the surviving subpopulation became resistant to up to 16 µg/mL cefiderocol/4 µg/mL avibactam (Figure 5e) representing the breakpoint concentration of each drug[22]. These data highlight how rapidly an HR isolate can develop a subpopulation of cells resistant to cefiderocol plus the addition of an effective beta-lactamase inhibitor; one exposure to sub-inhibitory concentrations just under the breakpoint concentrations selected for a population with elevated *blaADC-30* amplification and enhanced resistance to the combination. These data highlight how beta-lactamase gene amplification threatens both current beta-lactam/BLIs as well as those in the development pipeline.

DISCUSSION

In this work, we describe a mechanism for cefiderocol HR, making novel and broad insights into the dynamics of HR mediated by resistance gene amplification and how this flexible phenotypic phenomenon can impact rapid adaptation to new beta-lactams/BLIs without the necessity for stable evolution. Despite being developed in part to resist ESBLs, we found that cefiderocol can be inhibited by these beta-lactamases when their genes are amplified to high number in subpopulations of resistant cells. In response to chemical inhibition by BLIs or genetic inhibition, additional increases in the magnitude of gene amplification of these otherwise ineffective ESBLs facilitated survival of HR isolates treated with cefiderocol. This demonstrated that the relative activity of a beta-lactamase is a critical determinant of the extent of its gene amplification, and therefore that beta-lactamases thought to be overcome by a new beta-lactam or BLI could still pose a clinical problem. These findings change the landscape of our understanding of antibiotic resistance and highlight deficiencies in the current antibiotic development pipeline that allow HR to go undetected until it may potentially cause treatment failure in patients.

A clinical isolate exhibiting HR to a given antibiotic is often described as harboring a subpopulation of resistant cells. Interestingly, studying cefiderocol HR we observed that this subpopulation can actually be a continuum of resistant subpopulations exhibiting a spectrum of beta-lactamase gene amplification and resulting cefiderocol resistance. Cells with modest increases in the number of copies of a given beta-lactamase are much more abundant than those with the greatest levels of gene amplification which are present at the lowest frequency. For example, in *Enterobacter* strain RS, 1 in 5,000 cells have an average of 2 copies of *blaSHV-5*, while 1 in 150,000 cells have an average of 20 copies (Figure 1g). The generation of this continuum of resistant cells is likely dependent on at least two factors; 1) baseline activity of RecA which mediates homologous recombination through which gene amplification occurs, and over generations amplification in some cells can reach high number and 2) constraint of gene amplification due to the increased fitness cost of carrying such a high level of a beta-lactamase encoding amplicon[31]. It is important to note that the fitness cost may only partly be due to the beta-lactamase itself but could also be due to other genes within the amplified region.

It is interesting to consider the dynamics of the continuum of resistant cells in HR isolates and what this means for the overall flexibility and fitness of such strains. During exposure to a given beta-lactam, the cells with the minimum level of gene amplification that is sufficient to facilitate survival would predominate. In this way, the cells with the lowest fitness

cost possible are dominant. This strategy provides a strain with significant flexibility to balance survival and fitness. We expect this paradigm to be true for many of the multitude of genes capable of undergoing gene amplification across species and thus to be a foundational aspect of understanding HR. In addition to genes conferring resistance to beta-lactams, amplification of genes providing resistance to aminoglycosides[24,32] sulfonamides[32], tetracyclines[32] and polymyxins[33] have been identified in HR isolates.

Our findings of widespread cefiderocol heteroresistance paired with the apparent susceptibility of HR clinical isolates as classified by conventional antimicrobial susceptibility tests during the early testing of cefiderocol, suggest HR was overlooked in the development of cefiderocol. This prompted us to try to understand why cefiderocol HR was not detected. One of the first assays in the development of a new beta-lactam is to determine if it withstands the activity of existing beta-lactamases, as assayed using *in vitro* biochemical assays of the new beta-lactam and purified beta-lactamases. Significant data exists showing that cefiderocol resists the activity of most classes of beta-lactamases[7-9]. However, these assays using purified enzymes report enzymatic activity but do not take into account the possibility of beta-lactamase gene amplification. For example, if cefiderocol is 50-fold more resistant to ESBL activity and that is deemed sufficient and effective, this difference could perhaps be overcome functionally by extensive ESBL gene amplification.

The next and major assay used to evaluate the susceptibility of clinical isolates to a new beta-lactam is broth microdilution (BMD). In this assay, bacterial isolates are incubated with the beta-lactam in broth media and tested for growth to a visible optical density (cloudiness of the culture) by ~16 hours. However, we observed that the isolates studied here are classified susceptible by BMD and their heteroresistance is not detected. If the isolates are first incubated with cefiderocol, however, which selects for the resistant subpopulation, then the cefiderocol MIC by BMD is 16 or >64 µg/mL which is considered resistant (Extended Data Figure 8). These data indicate that when the frequency of the resistant subpopulation is extremely low (in this case ~1 in 10,000 cells), these resistant cells do not have sufficient time to grow to a visibly cloudy culture density in the 16 hour incubation time for BMD. In contrast, if their frequency is greatly enhanced by prior selection with cefiderocol, their growth in cefiderocol is readily apparent after BMD. This highlights how HR can often be undetected by BMD, and highlights an Achilles' heel of the beta-lactam development/testing process.

This leads to a model of the novel beta-lactam development pipeline that is undermined at multiple steps by HR (Figure 6). By design, cefiderocol was resistant to *in vitro* hydrolysis by

most beta-lactamases tested[7-9]. However, data presented in this manuscript demonstrate that beta-lactamase gene amplification can create a subpopulation of cells with enhanced resistance to cefiderocol. This population is present at low frequency, which evades detection by BMD, the standard clinical AST for cefiderocol accepted by CLSI[34]. This led to Phase III testing for the treatment of infections caused by isolates that appeared to be susceptible to cefiderocol but which resulted in relatively high rates of all-cause mortality. These high mortality rates correlated with the rates of cefiderocol HR in our clinical isolate collection[20,21]. During prolonged infection and treatment, we expect the resistant subpopulation to have sufficient time to grow and dominate the population, unlike the brief duration of growth before MIC determination by BMD. Thus, we suggest that undetected HR resulting from beta-lactamase gene amplification may contribute to treatment failure of cefiderocol, as well as future antibiotics that rely on the beta-lactam moiety.

Beyond the potential to mediate treatment failure of a beta-lactam to which a strain exhibits HR, we also investigated whether beta-lactamase gene amplification might contribute to adaptation to novel beta-lactams/BLIs to which a given HR isolate is initially susceptible. We observed that the BLIs clavulanate and avibactam could each render some HR isolates susceptible to cefiderocol (Figure 2b, 2d, 2f, Figure 3e, Extended Data Figure 5). We subsequently demonstrated that just one exposure of such a strain to a ¼ breakpoint concentration of cefiderocol/avibactam could lead to enhanced beta-lactamase gene amplification, leading to resistance to the breakpoint concentrations (Figure 5d-e). This shows how gene amplification can also mediate adaptation of a clinical isolate to a newly added BLI. Importantly, the clinical isolates studied here were collected before the clinical introduction of either cefiderocol or avibactam. Therefore, amplification of pre-existing beta-lactamases with suboptimal activity is a mechanism poised to mediate functional resistance and potential treatment failure of beta-lactams/BLIs which have not yet even been introduced into the clinic. Importantly, this mechanism of phenotypic flexibility does not rely on new stable evolution (e.g. of novel beta-lactamases with enhanced activity). These data highlight the paramount importance of screening for HR during the early stages of the drug development process, as well as during clinical susceptibility testing to avoid potential treatment failures.

DATA AVAILABILITY

Genome sequences will be available at NCBI Genbank with accession numbers CP096814-CP096817 (Mu1413), CP096894-CP096901 (Mu1956) and CP096818-CP096820 (Mu1984). The data supporting the findings of this study are available from the corresponding author on reasonable request.

COMPETING INTERESTS

DSW is listed as an author on a pending patent broadly related to this work.

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FIGURES

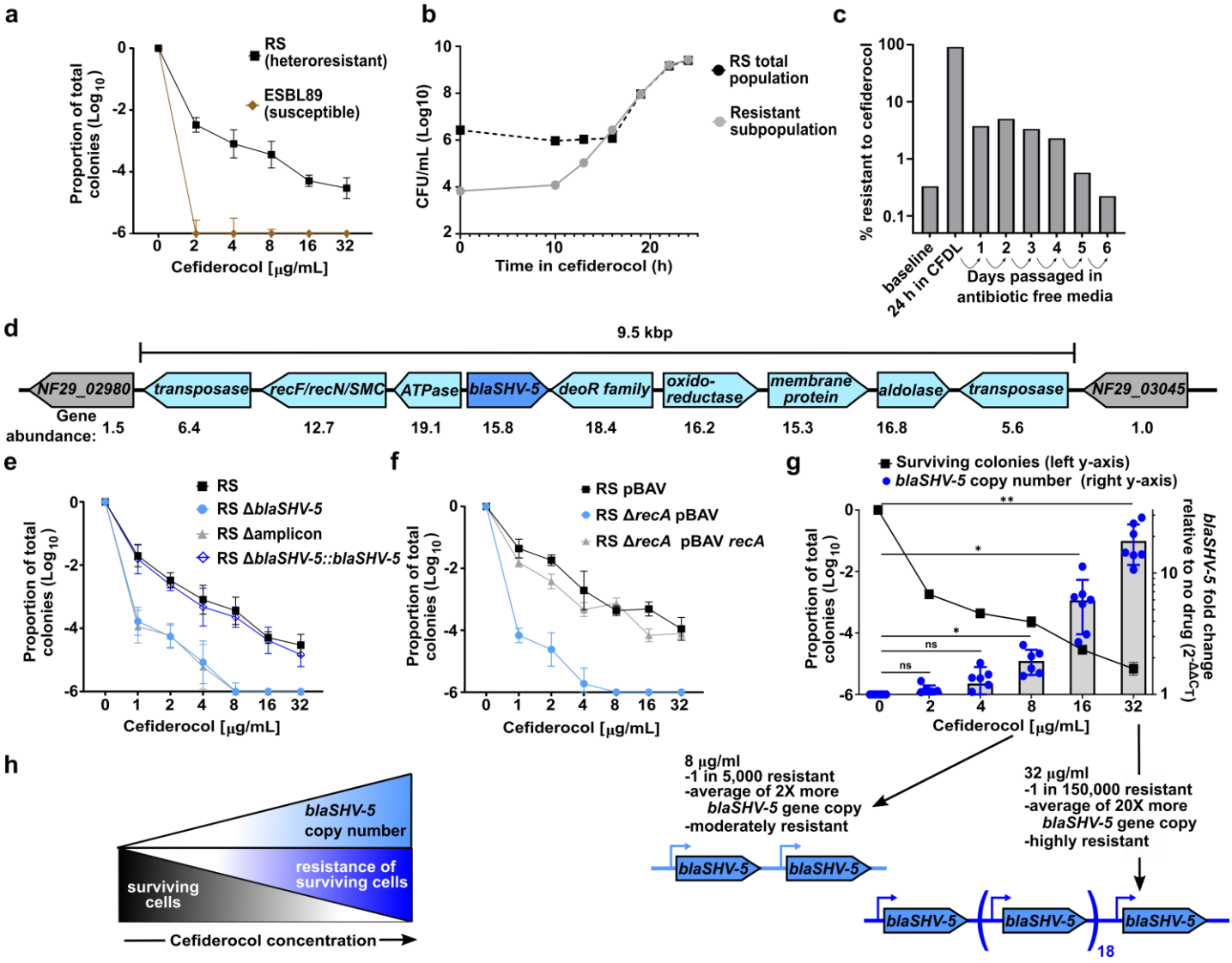

Figure 1. A cefiderocol resistant subpopulation of *Enterobacter cloacae* strain RS amplifies *blaSHV-5*. (**a**) Population analysis profile (PAP) of strains RS and *Klebsiella pneumoniae* ESBL89 plated on Mueller-Hinton agar (MHA) containing cefiderocol; the proportion of surviving colonies is quantified relative to MHA containing 0 cefiderocol, the mean and standard deviation are shown from two independent experiments with 3 biological replicates (RS) or 1 biological replicate (ESBL89) each shown. (**b**) Time kill of strain RS: growth in media containing 16 μg/mL cefiderocol over time and quantification of surviving colony forming units on MHA (RS total population) and MHA containing cefiderocol (resistant subpopulation). (**c**) Quantification of the cefiderocol resistant subpopulation of strain RS after growth in media alone (baseline), subcultured into cefiderocol and grown for 24 h, and subcultured every 24 h into fresh media without cefiderocol. At the end of each growth, an aliquot was diluted and plated onto MHA containing cefiderocol to quantify the resistant subpopulation. (**d**) The region of the RS chromosome amplified in cells surviving growth in cefiderocol is shown, with gene abundance shown below. (**e**) PAP on cefiderocol of RS, the Δ*blaSHV-5* and Δamplicon (Δ*NF29_02990-NF29_03045*) isogenic mutants, and the Δ*blaSHV-5* mutant complemented with *blaSHV-5* at the native site; the mean and standard deviation are shown from two independent experiments with 3 biological replicates each. (**f**) PAP of RS carrying empty vector (pBAV), the Δ*recA* isogenic mutant with pBAV, and the Δ*recA* mutant complemented with pBAV *recA* plated on cefiderocol. The mean and standard deviation from three independent experiments with 2 biological replicates each are shown. (**g**) PAP of RS plated on cefiderocol, with the proportion of surviving colonies in the black line on the left y-axis, with mean and standard deviation shown. *blaSHV-5* abundance was quantified from the samples collected at each concentration. Each dot indicates a biological replicate, and bars show the mean and standard deviation graphed on the right y-axis, from two independent experiments with 3-4 biological replicates each. * indicates $p<0.05$, ** $p<0.01$ by mixed-effects ANOVA analysis with Geisser-

Greenhouse correction and Dunnet's multiple comparison test. Below, details of the populations collected on 8 µg/mL or 32 µg/mL cefiderocol are shown. (**h**) A model of the RS population: as the concentration of cefiderocol increases, the proportion of the surviving cells decreases. The cefiderocol resistance of the surviving resistant cells increases with an increase in the *blaSHV-5* abundance.

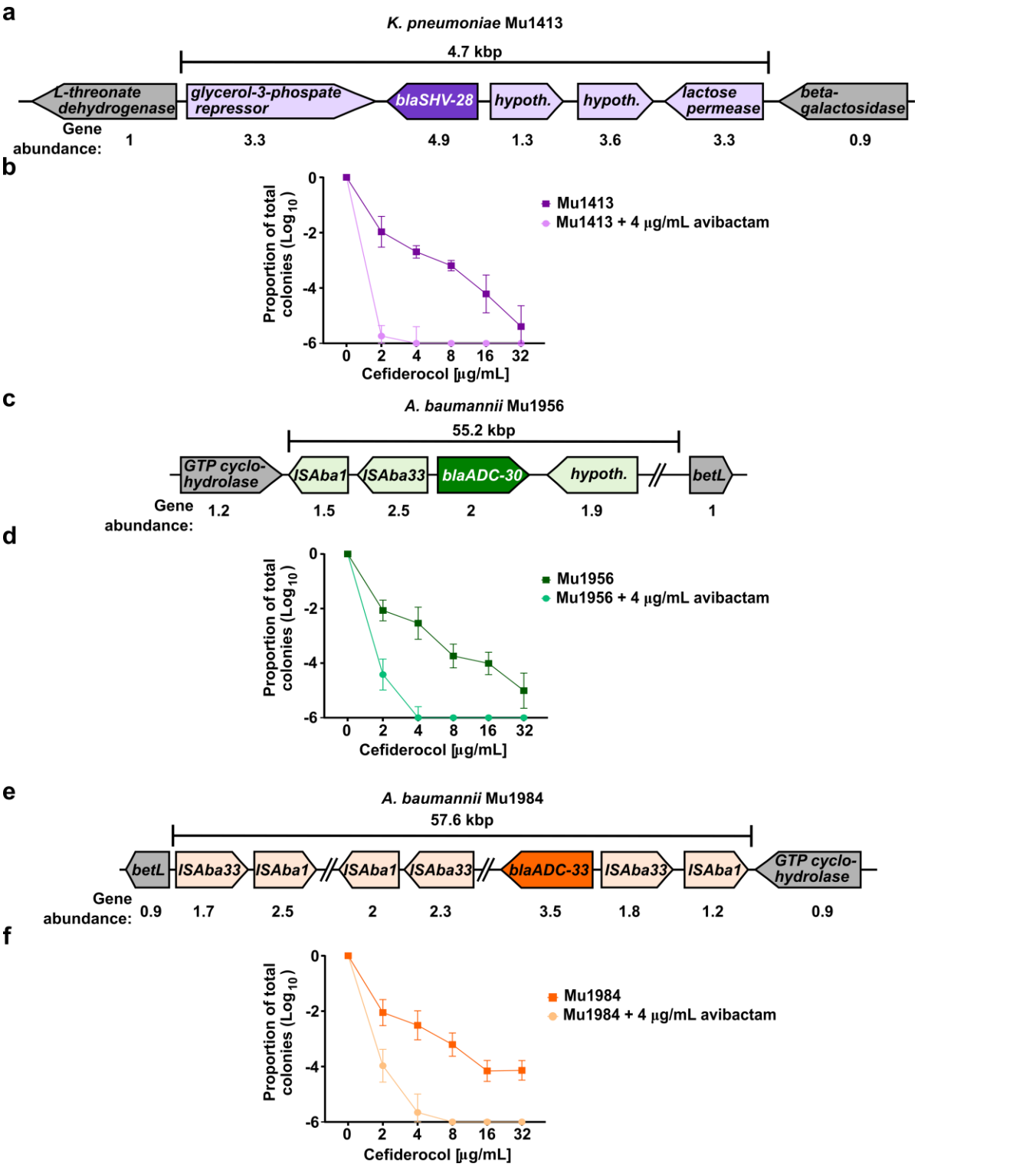

Figure 2. Cefiderocol resistant subpopulations of carbapenem-resistant isolates contain amplified regions encoding beta-lactamases. (**a**) The region of the *K. pneumoniae* Mu1413 chromosome with amplification in the cefiderocol resistant population is shown, with gene abundance shown below. (**b**) PAP of Mu1413 plated on cefiderocol or cefiderocol plus avibactam. (**c**) The region of the *A. baumannii* strain Mu1956 chromosome with amplification in the cefiderocol resistant population is shown, with gene abundance shown below. (**d**) PAP of Mu1956 plated on cefiderocol or cefiderocol plus avibactam. (**e**) The region of the *A. baumannii* strain Mu1984 chromosome with amplification in the cefiderocol resistant population is shown, as is gene abundance. (**f**) PAP of Mu1984 plated on cefiderocol or cefiderocol plus avibactam. For **b**, **d**, and **f**, the mean and standard deviation from two independent experiments with 3 or 6 biological replicates each is shown. In **c** and **e**, parallel lines indicate sequence regions omitted from the schematic.

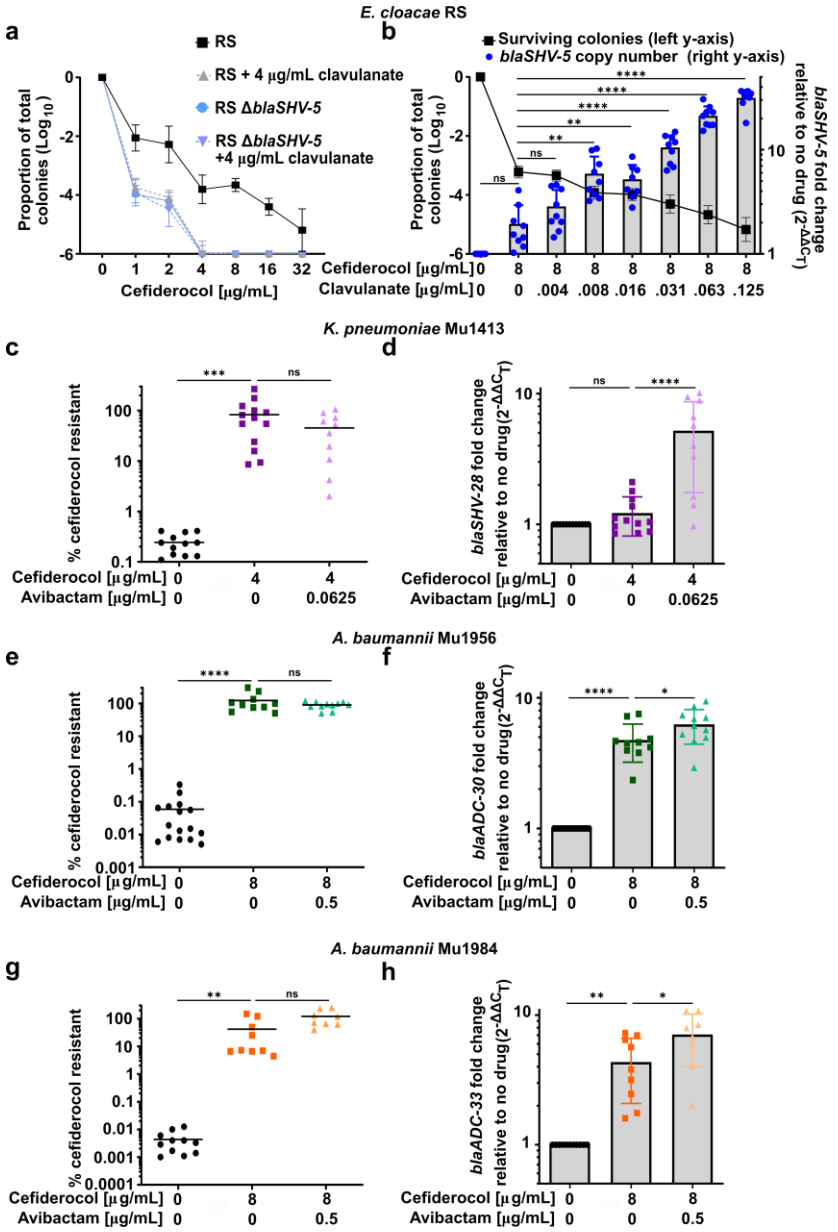

Figure 3. The magnitude of beta-lactamase amplification increases in the presence of beta-lactamase inhibitors. (**a**) PAP of strain RS or the isogenic Δ*blaSHV-5* mutant plated on cefiderocol or cefiderocol and 4 µg/mL clavulanate. The mean and standard deviation is shown from two independent experiments with 3 biological replicates each. (**b**) Strain RS was plated on MHA containing cefiderocol and clavulanate as indicated. The proportion of the surviving population is the line graph with the left y-axis. The corresponding gene abundance is graphed on the right y-axis, where each point indicates a biological replicate from two independent experiments with 3 or 6 biological replicates each and bars indicate mean and standard deviation. ** indicates p<0.01, **** p<0.0001 by mixed-effects ANOVA analysis with Geisser-Greenhouse correction and Dunnet's multiple comparison test (**c**) Quantification of Mu1413 survival on MHA containing 2 µg/mL cefiderocol after growth in media conditions indicated below the x axis. (**d**) Relative *blaSHV-28* abundance normalized to *cysG* of samples grown in broth in (**c**). (**e**) Quantification of Mu1956 survival on MHA containing 8 µg/mL cefiderocol after growth in media conditions indicated below the x axis. (**f**) Relative *blaADC-30* abundance normalized to *clpX* was quantified from the samples grown in broth in (**e**). (**g**) Quantification of Mu1984 survival on MHA containing 8 µg/mL cefiderocol after growth in media conditions indicated below the x axis. (**h**) Relative *blaADC-33* abundance normalized to *clpX* was quantified from the samples grown in broth in (**g**). For **c-h**, each symbol represents a biological replicate, with the mean indicated by a horizontal line. * indicates p<0.05, ** p<0.01, *** p<0.001, and ****p<0.0001 by one-way ANOVA with Sidak's correction for multiple comparisons. (**c-d**) are from two independent experiments with 4-6 biological replicates each, (**e-f**) from two independent experiments with 2-10 biological replicates each, and (**g-h**) from four independent experiments with 1-5 biological replicates each.

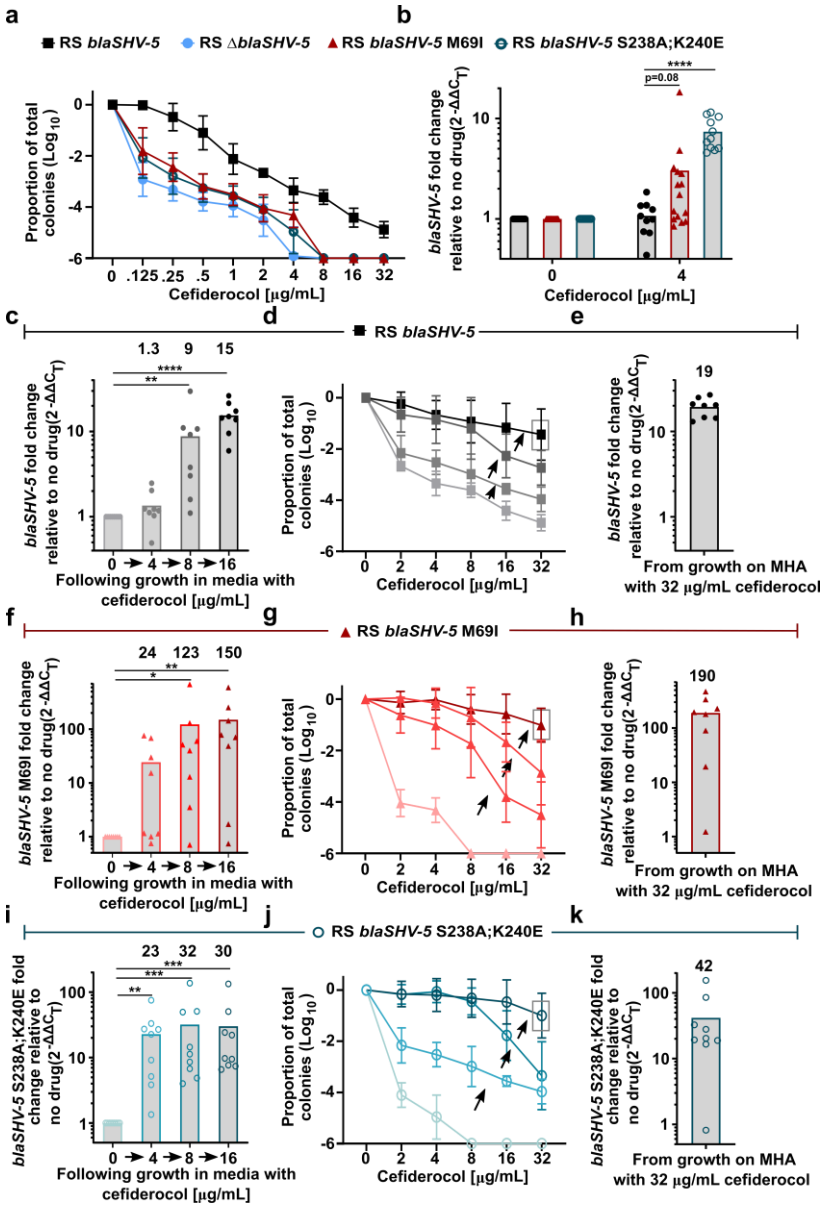

Figure 4. The magnitude of amplification inversely correlates with enzyme function. (**a**) PAP of *E. cloacae* RS strains plated on cefiderocol. Genotypes are Δ*blaSHV-5,* Δ*blaSHV-5*::*blaSHV-5* (referred to as *blaSHV-5*), Δ*blaSHV-5*::*blaSHV-5 M69I* (*blaSHV-5* M69I), and Δ*blaSHV-5*::*blaSHV-5 S238A;K240E* (*blaSHV-5* S238A;K240E; which is SHV-1). Shown are the means and standard deviation of three independent experiments with 3 biological replicates each. (**b**) *blaSHV-5* amplification from surviving colonies on 4 μg/mL cefiderocol in (**a**), each point indicates a replicate from three independent experiments, 10-15 replicates total for each strain.**** indicates p<0.0001 by two-way ANOVA with Sidak's correction for multiple comparisons. (**c**) *blaSHV-5* abundance after resistant colonies were collected on MHA containing cefiderocol and grown in broth containing the same concentration indicated. (**d**) PAP of samples from panel (**c**). (**e**) *blaSHV-5* fold change from colonies that survived on 32 μg/ml cefiderocol, indicated by gray box in (**d**). (**f**) *blaSHV-5* M69I abundance after resistant colonies were collected on MHA containing cefiderocol and grown in broth containing the same concentration indicated. (**g**) PAP of samples from panel (**f**). (**h**) *blaSHV-5* M69I fold change from colonies that survived on 32 μg/ml cefiderocol, indicated by gray box in (**g**). (**i**) *blaSHV-5* S238A;K240E abundance after resistant colonies were collected on MHA containing cefiderocol and grown in broth containing the same concentration indicated. (**j**) PAP of samples from panel (**i**). (**k**) *blaSHV-5* S238A;K240E fold change from colonies that survived on 32 μg/ml cefiderocol, indicated by gray box in (**j**). **c-k** show the mean from 3 independent experiments with 2-3 biological replicates each, with each dot indicating a biological replicate in **c**, **e**, **f**, **h**, **I**, and **k** and standard deviation shown in **d**, **g**, and **j**. For **c**, **f**, and **I**, * indicates p<0.05, ** p<0.01, *** p<0.001, and **** p<0.0001 by Kruskal-Wallis test with Dunnet's correction for multiple comparisons.

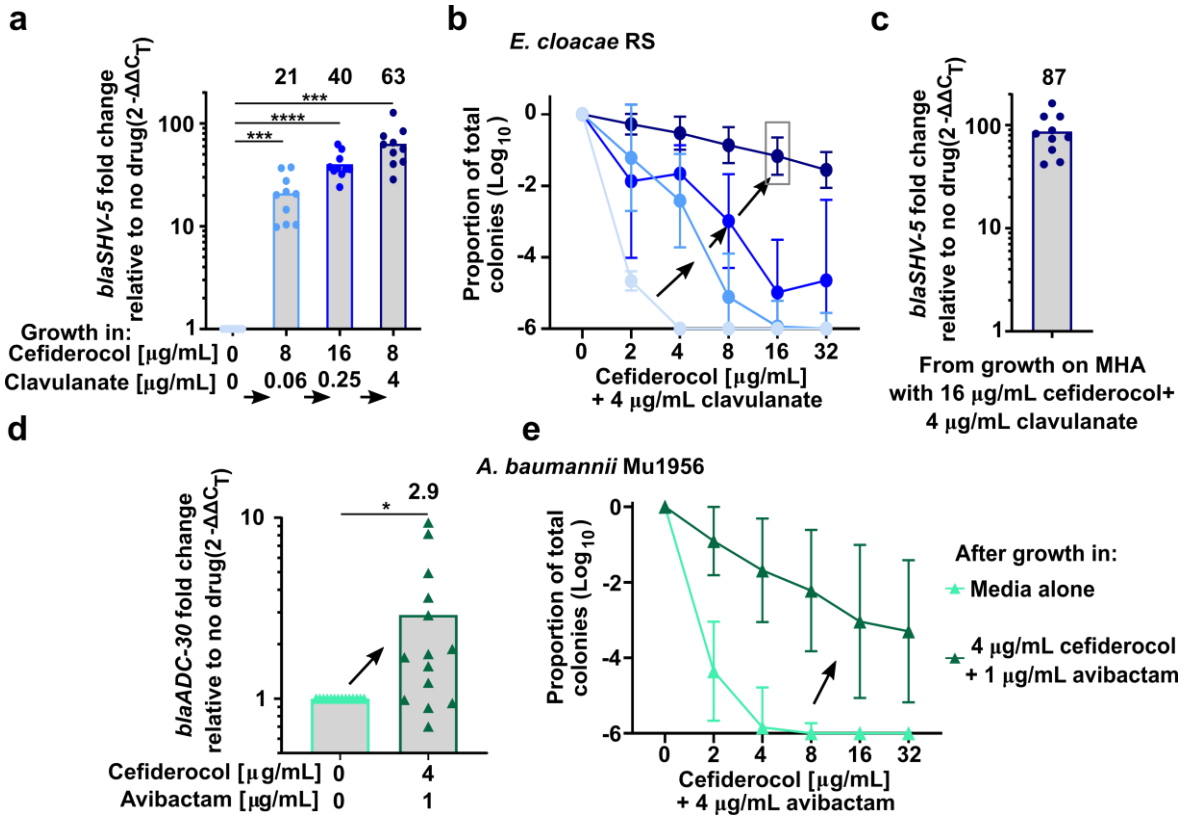

Figure 5. Cefiderocol/beta-lactamase inhibitor exposure increases amplification and results in a resistant subpopulation. (**a-c**) *blaSHV-5* abundance after serial exposure to cefiderocol/clavulanate. RS was plated on MHA alone or with 8 μg/mL cefiderocol and 0.06 μg/mL clavulanate and surviving colonies were collected and *blaSHV-5* abundance was quantified. A portion was subcultured into media with 8 μg/mL cefiderocol and 0.06 μg/mL clavulanate and grown for 24 h. The culture was plated on MHA containing 16 μg/mL cefiderocol and 0.25 μg/mL clavulanate. After 24 h, the resistant colonies were collected and *blaSHV-5* abundance was quantified. A portion was subcultured into media with 16 μg/mL cefiderocol and 0.25 μg/mL clavulanate and grown for 24 h. The culture was plated on MHA containing 8 μg/mL cefiderocol and 4 μg/mL clavulanate. After 24 h, resistant colonies were collected and *blaSHV-5* abundance was quantified. (**b**) PAP of RS plated on cefiderocol and 4 μg/mL clavulanate after growth in broth at each concentration of cefiderocol and clavulanate described in **a**. (**c**) *blaSHV-5* abundance from resistant colonies on 16 μg/mL cefiderocol and 4 μg/mL clavulanate [gray box in **b**]. (**d**) *blaADC30* abundance of Mu1956 after growth in broth containing cefiderocol and avibactam as indicated. (**e**) PAP of samples grown as indicated in **d**. **a-c** show the mean of results from two independent experiments with 5 biological replicates each, shown as individual dots in **a** and **c**, standard deviation is shown in **b**. For **a**, *** indicates p<0.001, and **** p<0.0001 by one-way ANOVA with Sidak's correction for multiple comparisons. **d-e** are from three independent experiments with 3-6 biological replicates each with the mean shown. For **d**, each dot indicates a biological replicate and for **e** standard deviation is shown. For **d**, * indicates p<0.05 by paired two-tailed t-test.

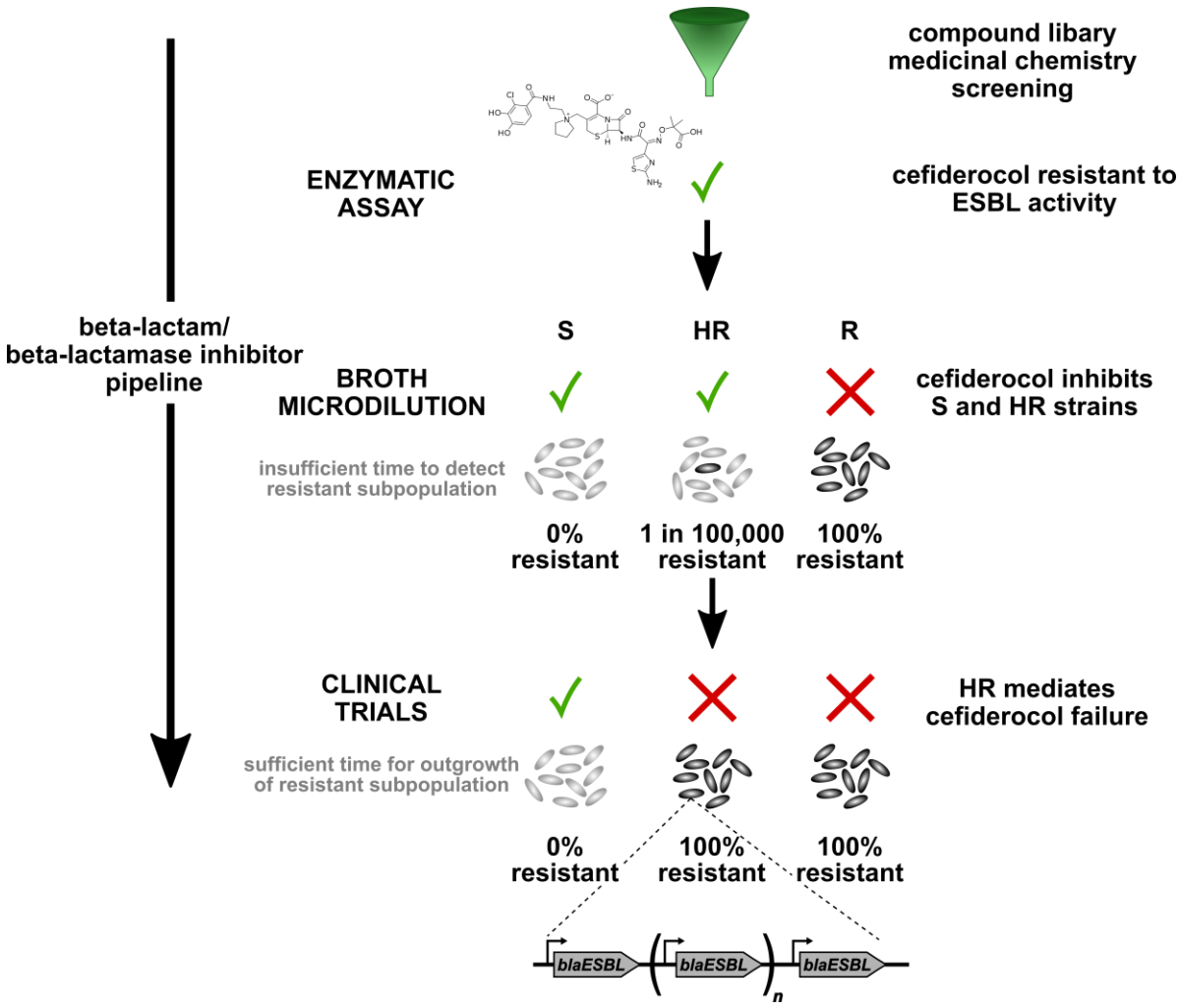

Figure 6. Heteroresistance threatens the beta-lactam/beta-lactamase inhibitor development pipeline. *In vitro* beta-lactamase enzymatic assays determined cefiderocol was resistant to extended-spectrum beta-lactamases (ESBL) and most carbapenemases tested. Broth microdilution (BMD) antimicrobial susceptibility testing assigned low cefiderocol minimum inhibitory concentrations (MIC) to both susceptible (S) and heteroresistant (HR) isolates, the latter of which harbored such a low frequency of resistant cells (e.g. 1 in 100,000) that they did not alter the overall MIC. BMD could detect only conventional resistance (R) in which 100% of the cells exhibit phenotypic resistance. HR is expected to cause treatment failure in patients because cefiderocol treatment selects for the resistant subpopulations which have amplifications of ESBL genes. These resistant cells become predominant during cefiderocol therapy and then can mediate treatment failure. In agreement with this model, unexpectedly high rates of treatment failure were observed in Phase III testing of cefiderocol, which correlated with rates of cefiderocol HR in surveillance studies. Taken together, the inability of the tests employed by the current antibiotic development pipeline (enzymatic assays and BMD) to detect HR leads drugs to which there are high rates of HR to progress to clinical testing where treatment failures may be observed. Incorporating testing for HR in the antibiotic development pipeline could potentially make the process more efficient and efficacious.

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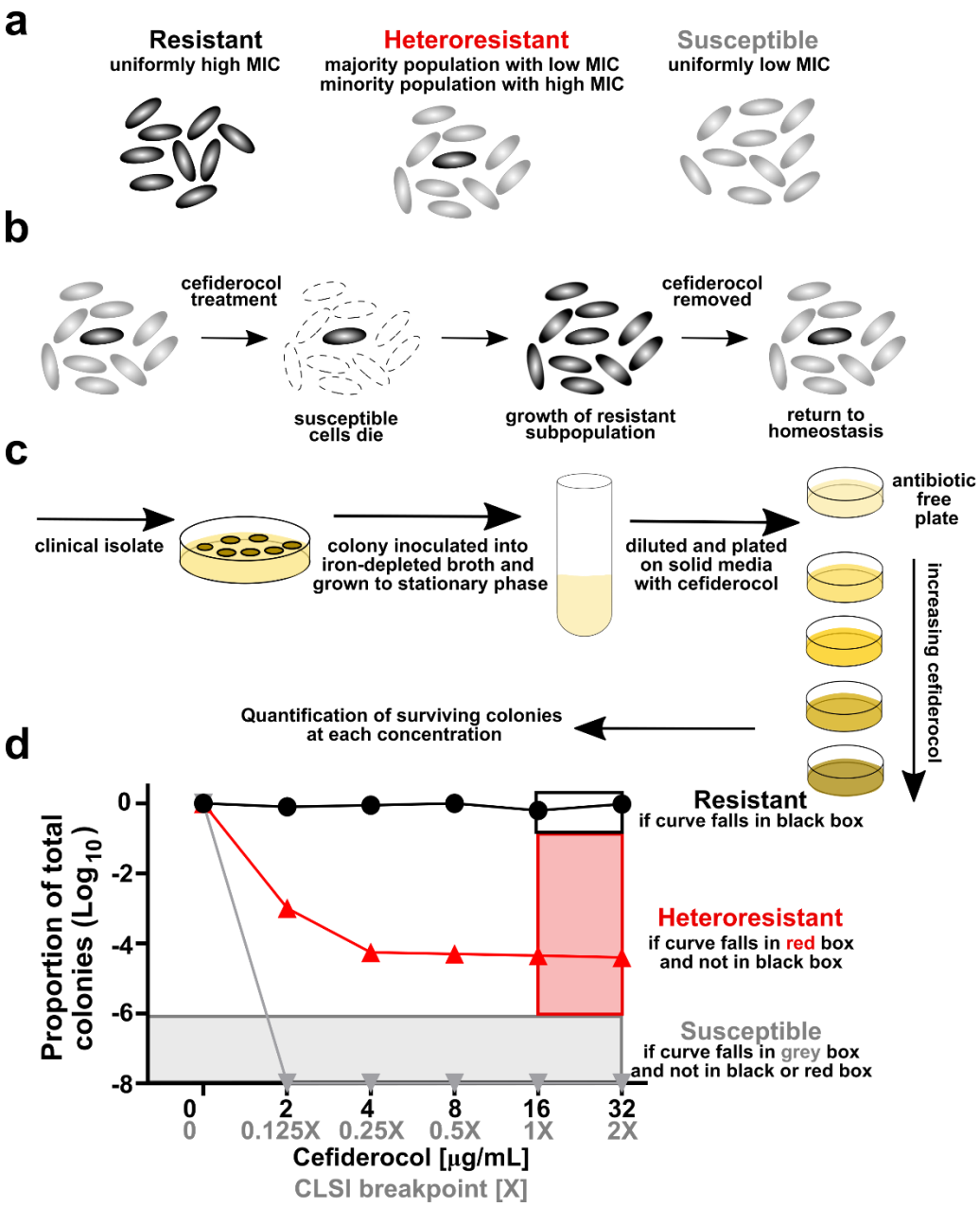
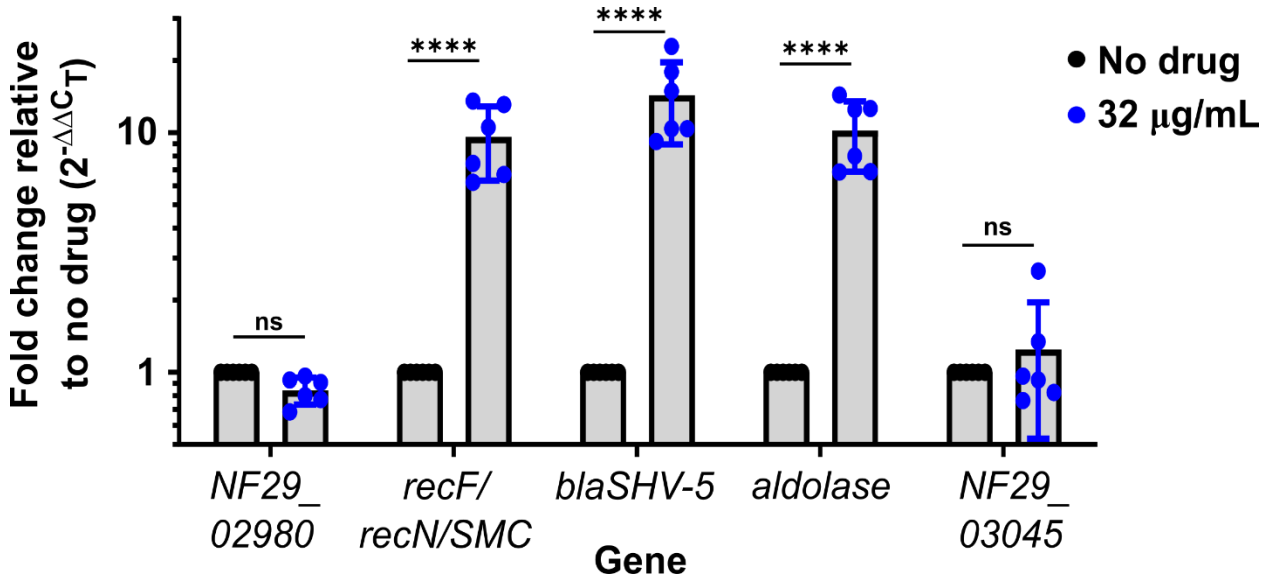
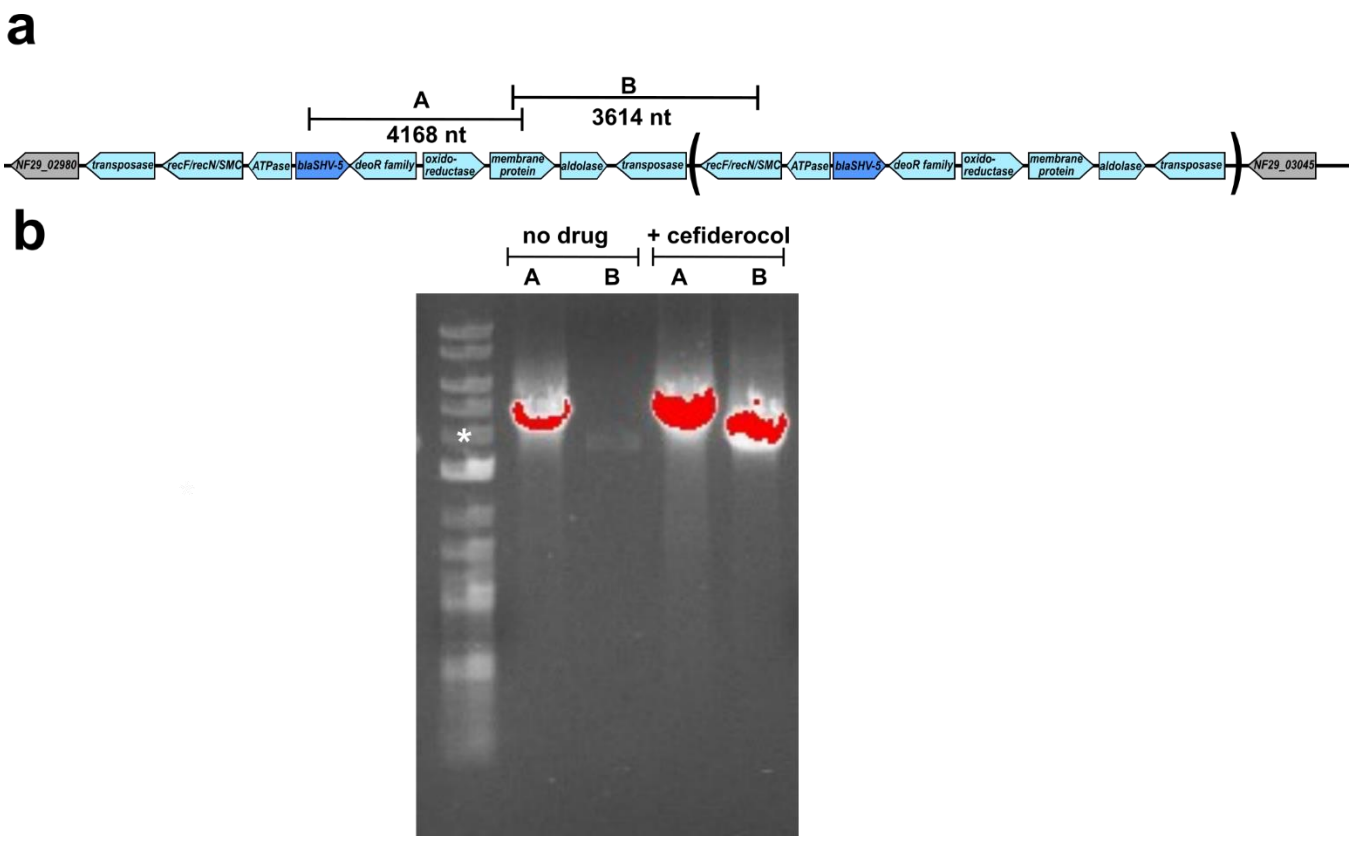
EXTENDED DATA FIGURES



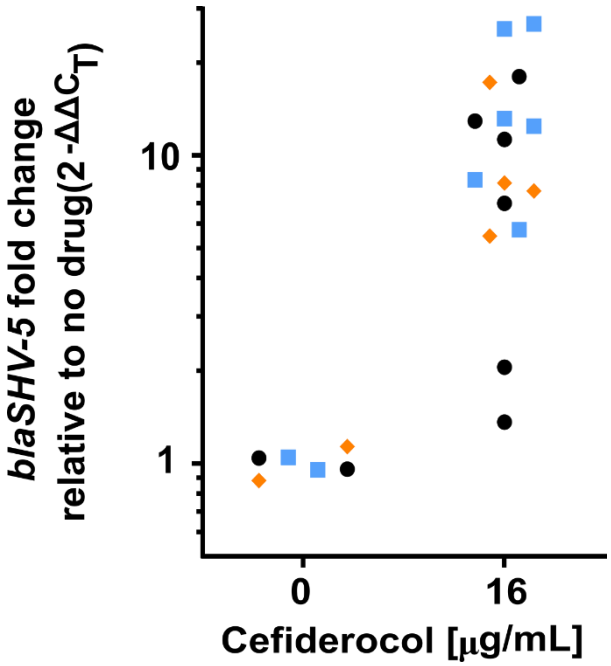
Extended Data Figure 1. Overview of heteroresistance and population analysis profile (PAP). (**a**) A depiction of the cells grown from a single colony of an isolate exhibiting conventional resistance, heteroresistance, or susceptibility to a given antibiotic. (**b**) Population dynamics of a heteroresistant isolate following cefiderocol treatment. (**c**) Cefiderocol PAP: a clinical isolate is isolated and grown in iron-depleted broth, and then serially diluted, plated, and grown on increasing concentrations of cefiderocol. (**d**) The surviving colonies are enumerated and the isolate is classified as resistant if at least 50% of the total colonies grow at 1 or 2X breakpoint. An isolate is considered susceptible if less than 0.0001% (-6 logs) of the cells grow at any concentration. An isolate is considered heteroresistant if there is less than 50% survival at 1X breakpoint and greater than 0.0001% at 1X and 2X breakpoint. Along the x-axis, the breakpoints based on CLSI are shown in gray, and the corresponding concentration of cefiderocol is in black.



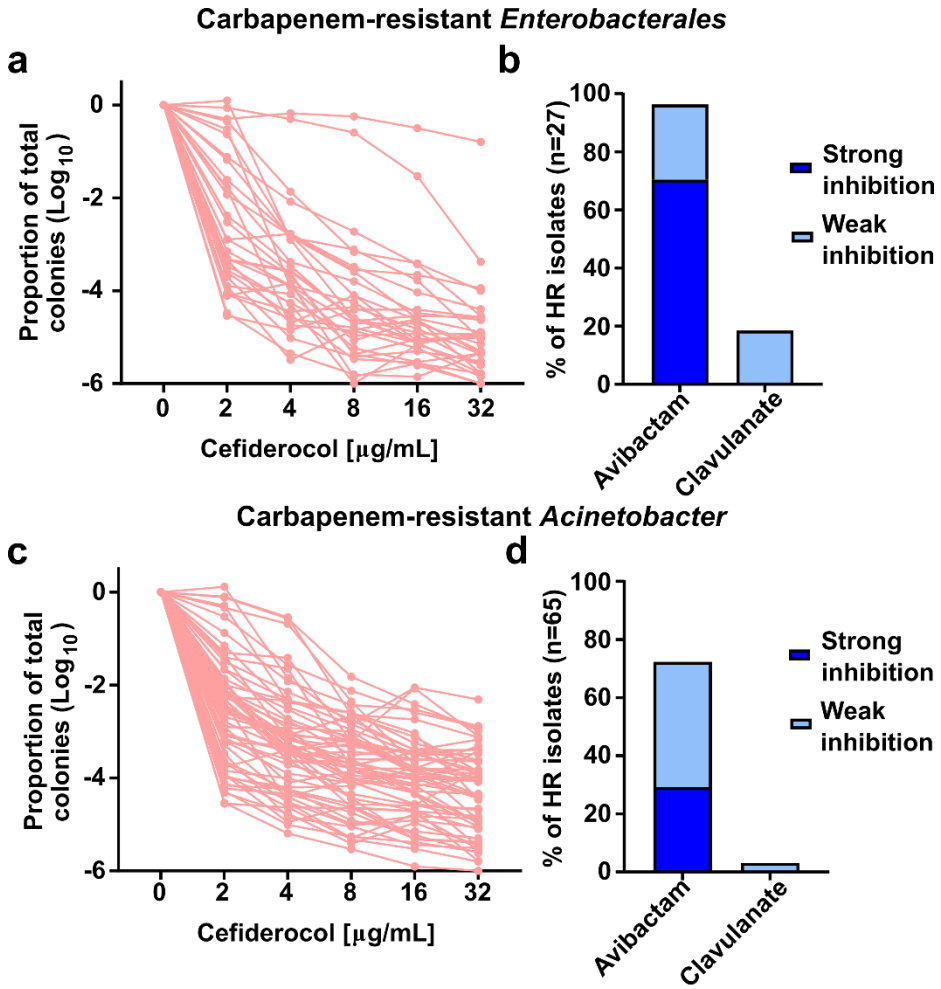
Extended Data Figure 2. Confirmation of genome sequencing gene abundance variation results by qPCR. Relative abundance of genes outside (*NF29_02980* and *NF29_03045*) and within (*recF/recN/SMC*, *blaSHV-5*, and *aldolase*) the amplified region normalized to *gyrA* (housekeeping gene) as quantified by qPCR from cells collected from Mueller Hinton agar containing no drug or 32 µg/ml cefiderocol. Shown are the means and standard deviation of two independent experiments with 3 biological replicates where each point indicates a biological replicate. **** indicates $p<0.0001$ by two-way ANOVA with Sidak's correction for multiple comparisons.



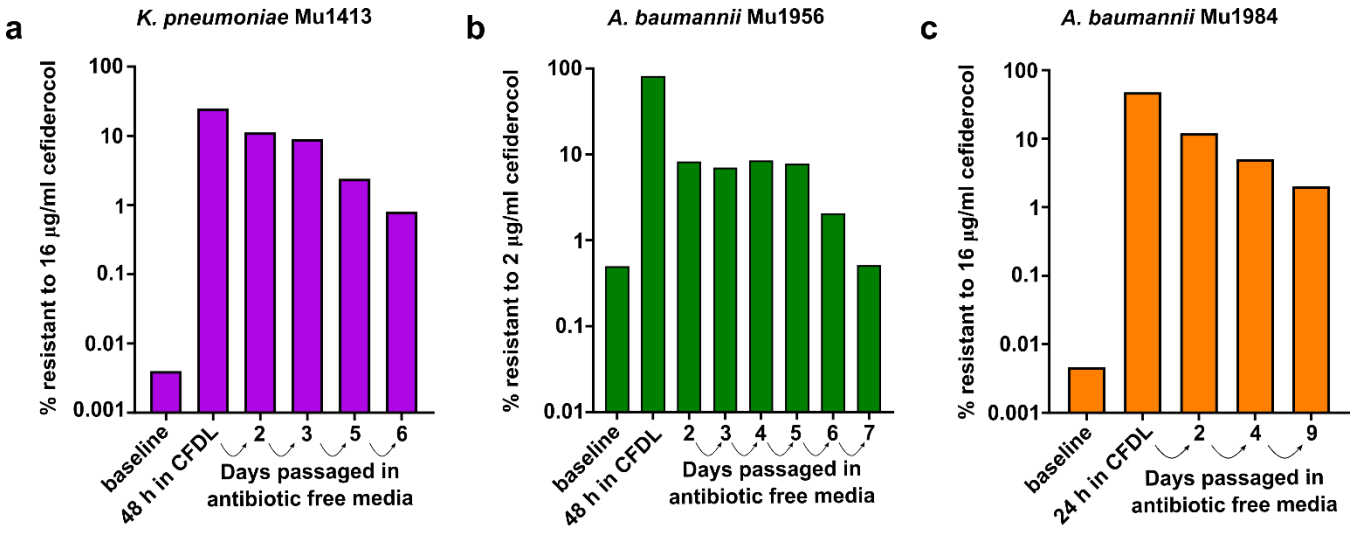
Extended Data Figure 3. PCR confirmation of *blaSHV-5* amplicon duplication. (**a**) A schematic of the predicted duplication of the *blaSHV-5* region in strain RS. Parentheses indicate duplication of the region with a single intervening copy of the transposase gene. PCR primer pair A is internal to the duplicated region, while primer pair B spans the duplication event. (**b**) PCR products in agarose gel from primer pairs A and B using DNA extracted following growth in media alone (second and third lane) or after growth in 32 µg/mL cefiderocol (fourth and fifth lanes) after 30 cycles of PCR. The asterisk indicates the 4 kb marker of the ladder. Note the faint product in lane 3 for primer pair B after growth in media alone which is consistent with a very small subpopulation of cells exhibiting gene amplification in the absence of cefiderocol.



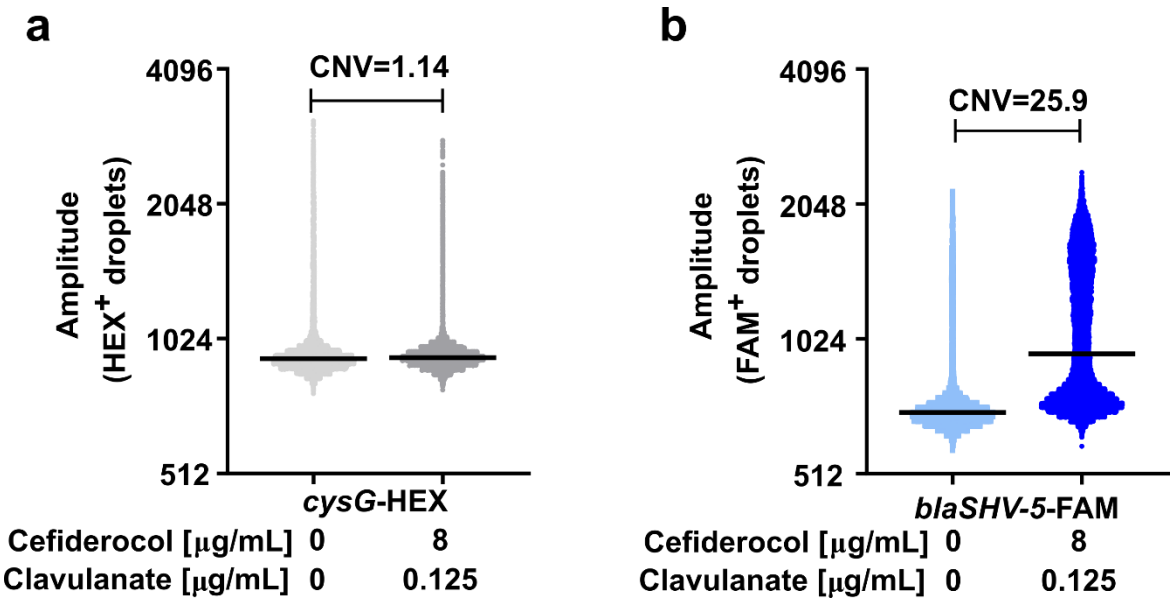
Extended Data Figure 4. Variation in *E. cloacae* RS *blaSHV-5* gene abundance. Quantification of strain RS *blaSHV-5* abundance normalized to *cysG* by qPCR. An overnight culture was diluted and plated on Mueller Hinton agar alone or containing 16 μg/mL cefiderocol. After growth, a single colony was selected, grown for 7 h in broth, cells collected, and DNA extracted. The ΔC_T of each replicate grown with no drug was averaged to calculate $\Delta\Delta C_T$. Each color is a separate biological replicate from a unique overnight culture, and each point is from a unique colony of the respective biological replicate.



Extended Data Figure 5. Cefiderocol heteroresistant CRE and CRAB clinical isolates are largely susceptible to avibactam inhibition. (**a**) Population analysis profile (PAP) of 27 carbapenem-resistant Enterobacterales (CRE) isolates identified as cefiderocol heteroresistant, where each line represents a unique isolate. (**b**) Results of PAP analysis for each isolate plated on cefiderocol plus avibactam or clavulanate. Inhibition was classified as “strong” if the presence of the respective beta-lactamase inhibitor caused the proportion of the surviving population to be below -6 log at ≤8 µg/mL cefiderocol, and was classified as “weak” if the presence of the inhibitor caused the proportion of the surviving population to be below -6 log at ≥16 µg/mL but not 8 µg/mL cefiderocol. The graphs are stacked bars. (**c-d**) Similar analyses as in A-B of 65 unique carbapenem-resistant *Acinetobacter baumannii* (CRAB) isolates.



Extended Data Figure 6. Resistance stability assays of representative carbapenem-resistant strains. (**a**) Quantification of the resistant subpopulation of *K. pneumoniae* Mu1413 after growth in media alone (baseline), subsequent passage in broth containing 8 µg/mL cefiderocol, and then sequential passages in antibiotic-free media. (**b,c**) Quantification of the resistant subpopulations of *A. baumannii* (**b**) Mu1956 and (**c**) Mu1984 in broth containing 32 µg/mL cefiderocol and subsequent passaging in antibiotic free media.



Extended Data Figure 7. Droplet digital PCR analysis of *blaSHV-5* abundance confirms qPCR results. Droplet digital PCR was performed on gDNA isolated as part of the experiment in Figure 3B, from *E. cloacae* RS grown on MHA containing 0 or 8 μg/mL cefiderocol and 0.125 μg/mL clavulanate. Each DNA sample was partitioned into ~20,000 droplets, PCR was performed, and amplification of the genes was detected for each droplet by quantifying the fluorescence of the HEX and FAM reporter probes. The probe internal to the PCR product amplifying (**a**) the housekeeping gene *cysG* was conjugated to the HEX fluorophore, and the (**b**) *blaSHV-5* probe was conjugated to the FAM fluorophore. Graphed are the raw amplitude values of each reporter from droplets containing both the HEX and FAM probes; >15,000 droplets per sample are shown. QuantaSoft Software uses a Poisson algorithm to quantify each gene as copies/μl input, and the copy number variation (CNV) is computed. The amplitude and CNV value for each pairwise comparison are shown above from a single replicate, representative of two biological replicates measured.

Isolate	MIC (µg/mL) No cefiderocol	MIC (µg/mL) + cefiderocol
E. cloacae RS	2	>64
K. pneumoniae Mu1413	2-4	16
A. baumannii Mu1956	4	>64
A. baumannii Mu1984	2	>64

Extended Data Figure 8. Broth microdilution does not detect cefiderocol resistant subpopulations. The results of cefiderocol broth microdilution of samples grown in broth only (no cefiderocol) or in broth with cefiderocol. Shown are the results from at least 3 biological replicates each.

METHODS

Isolate information

Carbapenem-resistant (CR) organisms were collected in Georgia, USA by the Georgia Emerging Infections Program as part of the CDC's EIP Multi-site Gram-negative Surveillance Initiative (MuGSI): *Acinetobacter baumannii* (CRAB, from 2012-2015) and *Enterobacterales* spp. (CRE, from 2011-2015). Identification of cefiderocol heteroresistance in these isolates was previously described and in Extended Data Figure 1[1]. *Enterobacter cloacae* RS is a clinical isolate previously described[2]. *K. pneumoniae* Mu1413 was submitted to NCBI previously as Biosample DHQP1604387 (identifier is MUGSI_426) but the genome was assembled de novo and uploaded to NCBI for this project separately; *A. baumannii* Mu1956 and Mu1984 genomes were assembled de novo and uploaded to NCBI (see Table 1).

Reagents

Mueller-Hinton agar (MHA; BD Difco), Mueller-Hinton broth (MHB; BD Difco), and cation-adjusted MHB (BD Difco) were used throughout. Iron-depleted cation adjusted Mueller-Hinton broth (ID-CA-MHB) was used for experiments with cefiderocol and prepared according to CLSI guidelines[3]: CA-MHB was treated with 1% chelex resin (Sigma-Aldrich) for 2 h at room temperature with gentle stirring, filtered to remove chelex, and 11.25 µg/mL $MgCl_2$, 22.5 µg/mL $CaCl_2$, and 10 µM $ZnCl_2$ were added back. Cefiderocol stock solution for experiments was prepared by incubating cefiderocol 30 µg disks (Hardy) with water at 37°C with shaking for 1 h to elute cefiderocol into solution at 420 µg/mL final concentration and subsequently filter sterilized, or by creating 10 mg/mL stock solution in DMSO of cefiderocol powder (MedChemExpress). Either stock preparation produced comparable results. For broth cultures, bacteria were cultured at 37°C with shaking.

Time kill

RS was streaked from -80 glycerol stocks to MHA for isolation and a single colony was used to start overnight cultures in ID-CA-MHB in 1.5 ml volume in 5 ml aeration culture tubes and grown for 10 h. Subsequently, 6 µl of this overnight culture was added to 3 ml ID-CA-MHB containing 16 µg/mL cefiderocol in 10 ml aeration culture tubes and grown for 24 h. At the timepoints indicated, 100 µl of culture was removed, serially diluted in PBS and plated on MHA containing 0 or 2 µg/mL cefiderocol, grown for 24 h and surviving colonies were enumerated.

Resistance stability assays

Strains were streaked from -80 glycerol stocks to MHA for isolation and a single colony was used to start overnight cultures in ID-CA-MHB in 1.5 ml volume in 5 ml aeration culture tubes and grown for 15-20 h. Cultures were back-diluted into 3 ml of fresh ID-CA-MHB in 10 ml aeration culture tubes and grown with cefiderocol for 24 h for RS and Mu1984 and for 48 h for Mu1413 and Mu1956 or without cefiderocol for 24 h for all strains. The conditions for each strain are listed in Table 1. Aliquots were taken to determine the percent resistance to cefiderocol to ensure selection of the resistant subpopulation (see Table 1). The remaining cells were collected and gDNA was extracted with Promega Wizard Genomic Extraction kit for whole genome sequencing. To confirm the resistant population selected in the presence of cefiderocol was the result of heteroresistance and not a spontaneous resistance mutation, the culture grown in cefiderocol was passaged each day by 1:1000 back-dilution into fresh ID-CA-MHB and grown for 24 h, with aliquots taken to quantify the resistant population and determine if the frequency substantially reduced after removal of antibiotic.

Whole genome sequencing

The samples from the resistance stability assays were subject to whole genome sequencing. The genome of the strains grown without antibiotic (mostly susceptible) was subjected to Illumina (650 Mbp) and Nanopore (300 Mbp ONT) sequencing to create a reference genome and quantify gene copy

variation. The genome of the resistant population from growth with cefiderocol was subject to Illumina sequencing and mapped to the respective reference genome with CNV analysis. Quality control and adapter trimming was performed with bcl2fastq[4]. Reads were mapped to their respective references via bwa mem[5]. PCR and optical duplicates were marked and excluded from the analysis using PicardTools' 'MarkDuplicates'[6] functionality. Aligned read counts were imported into R's CNOGpro[7]package. CNV events were called via CNOGpro using a bootstrapping methods, which calculates an average gene number event giving a possible upper and lower bound. The new reference genomes have been uploaded to NCBI (see Table 1). Genome sequencing and analysis were performed by Microbial Genome Sequencing Center (migscenter.com)

Population analysis profile

Population analysis profile (PAP) was performed as described previously[8] and indicated in Extended Data Figure 1. A given clinical isolate was grown overnight from a single colony streaked to MHA from -80°C glycerol stocks in 1.5 mL ID-CA-MHB. After approximately 16-20 h of growth, the culture was serially diluted in PBS in a 96-well plate (Falcon) and 7.5 or 10 µl of each dilution was plated on MHA containing antibiotics as indicated. Colonies were enumerated after 24-48 h of growth. Isolates were classified as resistant if the number of colonies that grew at the breakpoint concentration was at least 50% of those that grew on antibiotic free plates. If the isolate was not resistant, it was classified as heteroresistant if the number of colonies that grew at 2 or 4 times the relevant breakpoint was at least 0.0001% (1 in 10^6) of those that grew on antibiotic free plates. If the isolate was neither classified as resistant nor heteroresistant, it was classified as susceptible. The limit of detection is ~-7 logs, in the figures the y-axis is set at -6 logs of survival. See Extended Data Figure 1 for graphical representation.

In defining the features of heteroresistance in the four isolates of this work, based on four guidelines set forth by Andersson et al[9]:

1. Clonality: these isolate demonstrate monoclonal heteroresistance, they are purified isolates and single colonies are used throughout experiments
2. Level of resistance: the MIC of the resistant subpopulation in these strains is ≥8X the MIC of the main population, when comparing the amount of killing by the lowest concentration of cefiderocol used throughout, and the growth of the resistant subpopulation at 32 µg/mL.
3. Frequency of the resistant subpopulation: For the strains used throughout, the frequency at 2X the CLSI breakpoint is ~0.01% to 0.001%.
4. Stability: these isolates demonstrate unstable heteroresistance. As shown in the passage experiments after selection, there is a significant reduction in the resistant population frequency within 50 generations (~10 generations per passage) of growth in antibiotic free media.

RS confirmation of duplication

PCR was performed using GoTaq Master Mix (Promega) using 5 ng of DNA extracted from RS collected after growth on MHA containing 0 or 32 µg/mL cefiderocol, with 30 cycles of amplification. Primer pair A shown in Extended Data Figure 3 is JCP255/238 and primer pair B is JCP237/229 (Table 2). An equal volume of PCR product was loaded into 0.7% agarose containing ethidium bromide and imaged using Biorad Chemidoc XRS+ with Image Lab 6.1 software.

***E. cloacae* RS mutagenesis**

Lambda-red based allelic exchange was used to replace the *blaSHV-5* coding sequence with a kanamycin resistance gene, and then the gene was removed with Flp recombinase[10,11] to create unmarked in-frame deletions. The kanamycin resistance gene from pEXR6K_*kanFRT* was cloned using Promega GoTaq 2X master mix with Flp recognition sequence and homology to regions flanking the gene to be replaced using primers JCP293/244 for *blaSHV-5*, JCP269/271 for the amplicon region, and DH175/181 for *recA*. (Table 2). The purified PCR product was electroporated into competent RS pKD46-tet and transformants were selected on 90 µg/mL kanamycin. Transformants were re-streaked to Kan90 for isolation and subject to PCR for successful allelic exchange as indicated by a product size

change using primers flanking the gene to be replaced: JCP213/214 for *blaSHV-5*, JCP272/210 for the amplicon region, and DH179/182 for *recA* (Table 2). The mutants were then made electrocompetent and electroporated with pCP20[11]. Transformants were selected at 30°C on 50 µg/mL chloramphenicol, patched to 50 µg/mL chloramphenicol at 30°C and grown for 24 h, then patched to MHA and MHA+Kan90. Kanamycin-sensitive mutants were streaked for isolation and PCR was used with the same flanking primers to screen for the loss of the kanamycin resistance gene.

RS *recA* complementation

recA was cloned with Promega GoTaq 2X master mix with homology to the vector pBAV-T5 using primers JCP286/287. pBAV-T5-GFP[12] was amplified with primers JCP280/281 to remove the GFP gene. The amplified gene was assembled into pBAV-T5 using Hifi assembly (NEB) and propagated in *E. coli* NEB 5α with Kan35, then purified by plasmid miniprep (GeneJet). Plasmids were confirmed by sequencing with primers JCP339/340 by GeneWiz. Plasmids were transformed into electrocompetent strains with Kan90 selection. For experiments with complemented strains, Kan90 was included in ID-CA-MHB during broth culture but not in MHA plates.

E. cloacae RS *blaSHV-5* mutagenesis and allelic exchange

To mutagenize *blaSHV-5*, the region of the chromosome containing the coding sequence, and 750 bp upstream and 747 bp downstream were cloned using primers JCP343/344 into SmaI (NEB)-digested pTOX5 allelic exchange vector[13], using *E. coli* PIR2 and sequenced to confirm using primers JCP345/346/347/348 (GeneWiz). PIR2 carrying pTOX5 plasmids was propagated in MHB or MHA containing 20 µg/mL chloramphenicol and 2% glucose. The wildtype gene, the S238A;K240E variant, and the M69I variant was used to complement the Δ*blaSHV-5* deletion. S238A;K240E and M69I refers to the Ambler numbering system for beta-lactamases[14], in RS SHV-5 the numbering is S236A;K237E and M67I. The S238A;K240E mutations were created using the NEB Q5 Site-Directed Mutagenesis reagents according to the manufacturer using primers JCP323/324 and subsequently JCP325/326. M69I was created using primers JCP321/322. The resulting plasmids were sequenced with primers JCP213, JCP214, JCP345, and JCP346. The pTOX5 *blaSHV-5* vectors were transformed into electrocompetent *E. cloacae* RS Δ*blaSHV-5* with 25 µg/mL chloramphenicol and 2% glucose. Purple colonies were selected, resuspended in 2 ml LB+2% glucose, cells were collected when OD of culture reached approximately OD 0.2, the cells were washed twice in M9 salts + 2% rhamnose, and resuspended in 500 µl of M9 salts + 2% rhamnose. 200 µl and 20 µl were each plated on M9 agar+2% rhamnose and grown at 37°. After 36-48 h, non-purple colonies were restreaked to MHA. Allelic exchange was confirmed by PCR using primers JCP213/214, and primers JCP255, JCP256, and JCP258 were used for Sanger sequencing of JCP213/214 PCR product (GeneWiz). The chromosomal loci of each strain were 100% nucleotide match to the original chromosome except for introduced mutations.

Quantitative PCR

qPCR was performed from samples as described in each figure legend. DNA was purified using the Wizard Genomic DNA Purification kit (Promega) following the manufacturer's instructions. DNA was diluted to 10 ng/µl following quantification by Take3 (Biotek). Power SYBR Green PCR Master Mix (Applied Biosystems, Thermo Fisher) was used in 20 µl reactions with 10 ng of DNA and 50 nM primers. For each gene, multiple primer pairs were tested in advance of use according to guidelines of Applied Biosystems StepOnePlus Real-Time PCR machine to confirm primer pairs had similar efficiencies and melt curves. qPCR was performed in technical triplicate and fold change was calculated from the mean of technical triplicates. C_T values were normalized to the respective housekeeping gene. For all experiments except where noted, the fold change for each biological replicate was compared to the same replicate from no antibiotic conditions, resulting in a value of 1 for each no antibiotic replicate. Fold change was calculated using the $2^{-\Delta\Delta CT}$ method[15]. Primers are listed in Table 2.

RS was grown overnight in 1.5 ml ID-CA-MHB, serially diluted in sterile PBS, and 7.5-10 µl of each dilution were spot-plated on MHA. After ~24 h of growth at 37°, cells were collected. Surviving colonies from MHA containing no addition or various cefiderocol and potassium clavulanate (Combi-blocks) concentrations (as described in Figures) was collected with sterile cotton swab and resuspended in PBS, then cells were collected by centrifugation. For qPCR, *cysG* was detected with JCP241/242 and was used as the housekeeping gene for normalization, *blaSHV-5* with JCP257/258, NF29_02980 with JCP253/254, *recF/recN/SMC* (NF29_03000) with JCP251/252, *aldolase* (NF29_03030) with JCP237/238, and NF29_03450 with JCP265/266.

Mu1956 was grown overnight in 1.5 ml ID-CA-MHB and 12 µl were subcultured into 3 ml ID-CA-MHB alone or with addition of 8 µg/mL cefiderocol, or addition of 8 µg/mL cefiderocol and 0.5 µg/mL avibactam sodium (Combi-blocks), in a 10 ml volume aeration tube grown at a 45° angle with shaking. After 20 h, an aliquot was serially diluted and plated to MHA with and without cefiderocol addition and the rest of the cells were collected by centrifugation for DNA extraction. For qPCR *clpX* was detected with JCP302/303 and was used as the housekeeping gene for normalization, *blaADC-30* was detected with JCP298/299.

Mu1984 was grown overnight in 1.5 ml ID-CA-MHB and 12 µl were subcultured into 3 ml ID-CA-MHB with no addition or containing 8 µg/mL cefiderocol, 8 µg/mL cefiderocol and 0.5 µg/mL avibactam, in a 10 ml volume aeration tube grown at a 45° angle with shaking. After 20 h, an aliquot was serially diluted and plated to MHA with and without cefiderocol addition and the rest of the cells were collected by centrifugation for DNA extraction. For this strain, selection was less consistent and the samples for which <50% of the population grew on MHA containing 2 µg/mL cefiderocol were removed from analysis. For qPCR *clpX* was detected with JCP302/303 and and was used as the housekeeping gene for normalization*, blaADC-33* was detected with JCP337/338.

Mu1413 was grown overnight in 1.5 ml ID-CA-MHB and 3 µl were subcultured into 3 ml ID-CA-MHB with no addition or containing 4 µg/mL cefiderocol, 4 µg/mL cefiderocol and 0.0625 µg/mL avibactam, in a 10 ml volume aeration tube grown at a 45° angle with shaking. After 24 h of growth in media alone, or 48 h of growth in media containing cefiderocol, an aliquot was serially diluted and plated to MHA with and without cefiderocol addition and the rest of the cells were collected by centrifugation for DNA extraction. For qPCR *cysG* was detected with JCP318/319 and was used as the housekeeping gene for normalization, *blaSHV-28* was detected with JCP312/313.

Beta-lactamase Inhibitor screen

To screen carbapenem-resistant isolates with beta-lactamase inhibitors, the cefiderocol PAP was performed as above, except the MHA contained cefiderocol alone or with either 4 µg/mL clavulanate or 4 µg/mL avibactam. Each inhibitor was also included in MHA with no cefiderocol to confirm that the inhibitor alone did not affect total colony forming units.

ddPCR

Droplet digital PCR was completed at the Emory Integrated Genomics Core using the Bio-Rad QX200 ddPCR system using the QuantaSoft Analysis Pro Software. *blaSHV-5* primers were JCP257/258, and the custom probe internal to the PCR product was conjugated to FAM by Bio-Rad (#10031276) and cysG primers were JCP241/242, the custom prober internal to the PCR product was conjugated to HEX (#10031279), Black Iowa was the quencher. 100 ng of DNA was used in each well.

RS SHV-5 variant experiments

PAP was performed as described above but with cefiderocol from 0.125 µg/mL to 32 µg/mL for RS mutant strains: Δ*blaSHV-5*, Δ*blaSHV-5*::*blaSHV-5* (referred to as *blaSHV-5* in Figure 4), Δ*blaSHV-5*::*blaSHV-5* M69I (*blaSHV-5* M69I), and Δ*blaSHV-5*::*blaSHV-5* S238A;K240E (*blaSHV-5* S238A;K240E; which is SHV-1). Surviving colonies were collected from MHA containing 0 or 4 µg/mL cefiderocol and suspended in 1 mL sterile PBS, used for DNA extraction, and qPCR was performed as

described above. 10 µl of the suspension was added to 1 mL ID-CA-MHB containing 4 µg/mL cefiderocol. After 24 h growth in broth containing 4 µg/mL cefiderocol, PAP was performed. After 24 h of growth, surviving colonies were again collected from MHA containing 0 (this represents the gene abundance after growth in 4 µg/mL cefiderocol in Figure 4 panels c, f, and i) or 8 µg/mL cefiderocol. From the suspension of cells collected from 8 µg/mL cefiderocol, 10 µl of the suspension was added to 1 mL ID-CA-MHB containing 8 µg/mL cefiderocol. After 24 h growth in broth containing 8 µg/mL cefiderocol, PAP was performed. After 24 h of growth, surviving colonies were again collected from MHA containing 0 (this represents the amplification number after growth in 8 µg/mL cefiderocol in Figure 4 panels c, f, and i) or 16 µg/mL cefiderocol. From the suspension of cells collected from 16 µg/mL cefiderocol, 10 µl of the suspension was added to 1 mL ID-CA-MHB containing 16 µg/mL cefiderocol. After 24 h growth in broth containing 16 µg/mL cefiderocol, PAP was performed. After 24 h of growth, surviving colonies were again collected from MHA containing 0 (this represents the amplification after growth in 16 µg/mL cefiderocol in Figure 4 panels c, f, and i) or 32 µg/mL cefiderocol and was used for DNA extraction and qPCR was performed (Figure 4 panels e, h, and k). For qPCR, the surviving colonies collected from MHA containing no cefiderocol in the initial PAP is the comparator for calculating $\Delta\Delta C_T$.

RS cefiderocol/clavulanate passage

PAP was performed as described above on cefiderocol with 4 µg/mL clavulanate, as well as plated on MHA containing 8 µg/mL cefiderocol and 0.06 µg/mL clavulanate. After ~24 h of growth, surviving colonies were enumerated for each replicate and collected from MHA containing no cefiderocol (the baseline comparator for qPCR). The surviving colonies from MHA containing 8 µg/mL cefiderocol and 0.06 µg/mL clavulanate were resuspended in 1mL PBS from which 7.5 µl was inoculated into 2 mL ID-CA-MHB with 8 µg/mL cefiderocol and 0.06 µg/mL clavulanate and the remaining cells were collected for DNA extraction. After 24 h of growth, PAP was performed on cefiderocol with 4 µg/mL clavulanate and the culture was also plated on MHA containing 16 µg/mL cefiderocol and 0.25 µg/mL clavulanate. After 24 h, surviving colonies were enumerated and collected from MHA containing 16 µg/mL cefiderocol and 0.25 µg/mL clavulanate and resuspended in 1ml PBS. 7.5 µl was added to 2 mL ID-CA-MHB with 16 µg/mL cefiderocol and 0.25 µg/mL clavulanate and the remaining cells were collected for DNA extraction. After 24 h of growth, PAP was performed on cefiderocol with 4 µg/mL clavulanate and the culture was also plated on MHA containing 8 µg/mL cefiderocol and 4 µg/mL clavulanate. After 24 h, surviving colonies were enumerated and collected from 16 µg/mL cefiderocol and 4 µg clavulanate for qPCR.

Mu1956 passage experiment

Mu1956 was grown overnight and PAP performed on MHA containing 0-32 µg/mL cefiderocol and 4 µg/mL avibactam. From the overnight culture, 12 µl were inoculated into 3 ml ID-CA-MHB without drug or with 4 µg/mL cefiderocol and 1 µg/mL avibactam, in a 10 ml volume aeration tube grown at a 45° angle with shaking. The cells of the overnight broth culture collected for qPCR. After 24 h, PAP was performed on MHA containing cefiderocol and 4 µg/m avibactam. The cells of the broth cultures were collected for qPCR.

Broth Microdilution

Broth microdilution was used to determine the minimum inhibitory concentrations (MICs) of cefiderocol for strains grown in ID-CAMHB with and without cefiderocol by following the CLSI protocol[16]. RS was grown for 24 h in 0 or 32 µg/mL cefiderocol, and Mu1413, Mu1984, and Mu1956 were grown for 24 h in 0 or 8 µg/mL cefiderocol. In brief, a suspension of bacteria was diluted in ID-CA-MHB to $5x10^4$ CFU/well; cefiderocol concentrations were prepared in 2-fold dilutions ranging from 0.0125 to 64 µg/mL. After incubation of 16 hours (or 20 hours for *A. baumannii*) at 37°C, MICs were determined according to the well with the lowest concentration in which bacterial growth was not visible.

Table 1. Genome sequencing information				
Strain	**Mu1413**	**Mu1956**	**Mu1984**	**RS**
NCBI accession number	CP096814-CP096817	CP096894-CP096901	CP096818-CP096820	CP010512[2]
NCBI Biosample ID	SAMN27934724	SAMN27988045	SAMN27957725	N/A
Nucleotide position of amplified region	2362116-2366787	2832530-2888192	1116907-1174529	615067-624588
Average gene copy of amplified region in cefiderocol treated genome	3.6	1.88	3.37	15.0
Average gene copy of amplified region in untreated genome	0.94	-	0.97	1.05
Back-dilution in cefiderocol containing broth	1:1000	1:100	1:250	1:250
Concentration of cefiderocol in broth selection	8 µg/mL	32 µg/mL	32 µg/mL	32 µg/mL
% resistant in untreated population on MHA	0.05% resistant to 8 µg/mL, 0.004% resistant to 16 µg/mL	0.12% resistant to 2 µg/mL	0.03% resistant to 8 µg/mL, 0.05% resistant to 16 µg/mL	0.09% resistant to 2 µg/mL
% resistant in cefiderocol treated population on MHA	62% resistant to 8 µg/mL, 25% resistant to 16 µg/mL	81.8% resistant to 2 µg/mL	76.2% resistant to 8 µg/mL, 47.6% resistant to 16 µg/mL	100% resistant to 2 µg/mL

TABLE 2. Primers	
ID	**SEQUENCE**
JCP255	CTGGGAAACGGAACTGAATG
JCP238	TGCCGAGGGTAAATATCAGC
JCP237	ACTGTATCCGCCCCTTTACC
JCP229	AGGCTTTCGGGGTTTATCAG
JCP293	GGGCGTGGTGATCGGCAAACAGCGACGCCCGACAGAGTGCGGTATTTAGTGAGGTCGACGGTATCGATAA
JCP244	CACGCCTGCGAGGTGCTGCGGGCCGGATAACGCGCGCGGCCACCGCCGGGGCATAGCTGCAGGATCGATA
JCP269	GGTTGCATAGACAACACGATGGACCCACGCATGAGCTTCAAGTCTGTTATGAGGTCGACGGTATCGATAA
JCP271	TGGCCTTTGACATCGTAGCCAGATTCTACCACTGAAAGAGGGACGCAATTGCATAGCTGCAGGATCGATA
DH175	GAAATCCACGGTGAAGCACAGTCGCTTCTCCCGGCATGACAGGAGTAATAGAGGTCGACGGTATCGATAA
DH181	AGAAATGCAAAAAGGGCCGCATCAGCAGCCCTTCATTTTAAACGAAAGAAGCATAGCTGCAGGATCGATA
JCP213	CCCACCAGCATGACCTTAAT
JCP214	GGGATATTCACCCCTGATGA
JCP272	CACAAACCTCAGACCTGAGT
JCP210	GAAATCAAAACACCGCCTGT
DH179	ACGCTCTGGCAACAATTTCT

DH182	TGTTCACTGTGGTCACGAACGGCCAGGATG
JCP286	ACTAGAGAAAGAGGAGAAATACTAGATGGCTATCGACGAAAAC
JCP287	CCTGCAGCGGCCGCTACTAGTATTATTAAAAGTCTTCGTTGGTTTC
JCP280	TAATACTAGTAGCGGCCG
JCP281	CTAGTATTTCTCCTCTTTCTC
JCP339	GACGAACTCCAATTCACTGTTCCTTGC
JCP340	GGAGAGCGTTCACCGACAAACAACAG
JCP343	AATTAATTTAAATGCATCCC*GGG*AAATATTGGATTTACCGCAGCCGTTAGG
JCP344	GATCGAGCTCGAGACGTCCC*GGG*CCTGGTGGAGCAGCGTGG
JCP345	TCGCAAACCTGTCGTAGC
JCP346	AGATCCTTGGCGGCAAGAAA
JCP347	GGAAACGGAACTGAATGAGG
JCP348	TGGATGCCGGGACTACC
JCP323	GACCGGAGCTGCCAAGCGGGGTG
JCP324	TTATCGGCGATAAACCAGC
JCP325	GACCGGAGCTGGCGAGCGGGGTGCGC
JCP326	TTATCGGCGATAAACCAGCCCGC
JCP321	CGTTCATCGGCGCGCCAG
JCP322	CTTTCCCATGATTAGCACCTTTAAAGTAGTGCTC
JCP256	GCTTGCTAGCTCCGGTCTTA
JCP258	CGCTGTTATCGCTCATGGTA
JCP241	GCTTTATCATGCCGTCGATT
JCP242	CAGCCTGTCGTTCACAAAGA
JCP257	CTTTCCCATGATGAGCACCT
JCP253	CTCGGAAGAGATTGTGATTGC
JCP254	TCAATTCTTTCCACGTGAGC
JCP251	CCAATCGCGAATTCATGATA
JCP252	GTTTCGCTCTGAAAATGCGT
JCP265	ATTTCGGTTGTGGCTTTGTC
JCP266	AACTTTCCGTCCGGGATAAT
JCP302	TCGTGACGTGTCTGGTGAAG
JCP303	CAGTCCAGCAAATGCACCAC
JCP298	CAGGTATGGCTGTGGGTGTT
JCP299	TGCATATCCACCTGCTGTCG
JCP337	CAGGTATGGCTGTGGGTGTT
JCP338	TGCATATCCACCTGCTGTCG
JCP318	GGGTTAAGCAGCAGTTTGCC
JCP319	AACTGCTCAGTGGTATCCGC
JCP312	ATCTCCCTGTTAGCCACCCT
JCP313	TGCTCATCATGGGAAAGCGT

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