

1 **TITLE:**

2 Epistasis drives rapid divergence across multiple traits during the adaptive evolution of
3 a carbapenemase

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14 **ABSTRACT:**

15 Interactions among beneficial mutations (i.e., epistasis) are often strong enough as to
16 direct adaptation through alternative mutational paths. While alternative solutions
17 should display similar fitness under the primary selective conditions, their properties
18 across secondary environments may differ widely. The extent to which these cryptic
19 differences are to be expected is largely unknown, despite their fundamental and
20 practical importance, such as in the search for exploitable collateral sensitivities among
21 antibiotic resistance mutations. Here we use directed evolution to characterize the
22 diversity of mutational paths through which the prevalent carbapenemase KPC-2 can
23 evolve high activity against the clinically-relevant antibiotic ceftazidime, an initially
24 poor substrate. We identified 40 different substitutions, including many common clinical
25 settings, spread along 18 different mutational trajectories. Initial mutations determined
26 four major groups into which the trajectories can be classified, a signature of strong
27 epistasis. Of note, despite minor variation in final ceftazidime resistance, groups
28 diverged markedly across multiple phenotypic dimensions, from molecular traits such
29 as stability and hydrolytic efficiency to macroscopic traits such as growth rate and
30 activity against other β -lactam antibiotics. Our results indicate that cryptic yet
31 consequential phenotypic differences can readily accumulate under strong selective
32 pressures, bearing implications for efforts to prevent unwanted evolution in microbes.

33

34

35 INTRODUCTION

36

37 Adaptation to strong selection pressures inevitably reduces genetic diversity. The
38 fixation of adaptive alleles reduce variation not only at the locus where they occur, but
39 also at all other genomic regions that are not separated from this locus by
40 recombination¹; a phenomenon referred to as a “selective sweep”^{2,3}. Early models
41 assumed that beneficial mutations were exceedingly rare, so selective sweeps would
42 typically have a single origin and diversity would be drastically reduced (i.e., a “hard
43 sweep”)^{4,5}. Recent work, however, emphasizes that many natural populations can be
44 large enough as to make beneficial mutations common, especially in microbes^{6,7}. As a
45 result, adapted populations often consist of multiple genotypes carrying alternative
46 solutions to the same adaptive challenge (i.e., a “soft sweep”)^{8,9}. The extent to which
47 this genetic variation can have evolutionary consequences beyond the local selective
48 conditions is crucial to many fundamental and applied problems in ecology and
49 evolution. Examples include understanding biodiversity patterns along environmental
50 gradients^{10,11}, the tempo and mode of divergence^{10,11} and incipient speciation^{12,13}, and the
51 capacity of populations to adapt to new challenges^{14,15}, crucial both for conservation
52 efforts and for pest and pathogen control¹⁶.

53

54 A scenario in which this issue is particularly relevant is the fight against antibiotic
55 resistance evolution, a major threat to public health worldwide¹⁷. Next-generation
56 sequencing has revealed that bacterial populations adapted to antibiotics, both in
57 experimental and clinical settings, are often comprised by multiple clones with different
58 resistance determinants^{6,18-20}. While presumably equivalent in fitness under the primary
59 selective conditions, these different alternatives may display distinct liabilities and
60 strengths across other clinically relevant conditions. This possibility has direct relevance
61 to approaches that seek to apply thinking to prevent or eliminate antibiotic-resistant
62 variants²¹⁻²⁴. One such approach is prioritizing antibiotics for which resistance comes at
63 a substantial fitness cost, either by reducing growth rates, transmission, or virulence, or
64 by increasing sensitivity to the immune system or the host's internal environment²⁵.
65 Another, more recently advocated strategy is the use of drug combination or cycling
66 regimes that exploit trade-offs among antibiotics (i.e., collateral sensitivity), based on

67 the common observation that resistance mutations to a given drug sometimes induce
68 hypersusceptibility to other drugs^{26,27}.

69

70 While promising, the success of these strategies in clinical practice hinges on the
71 assumption that evolutionary outcomes are largely predictable; an assumption that
72 cannot be taken for granted, especially under the variable conditions of real-world
73 applications^{28–30}. In fact, our current understanding of evolutionary genetics raises at
74 least two important cautionary points to be considered. First, as many authors have
75 already pointed out, resistance mutations are rarely unique or functionally identical;
76 instead, they are better depicted as a pool of different classes with varying frequencies
77 and phenotypic profiles^{28,31–33}. As a consequence, the behavior of a “typical” mutant may
78 not represent the entire distribution it comes from. Moreover, changes in population
79 genetic parameters can alter which subset of mutations can be deemed as “typical”:
80 experiments show that variations in population size, mutation bias and selection strength
81 can result in different subsets of mutations becoming preferentially substituted during
82 adaptation^{6,28,34–37}.

83

84 A second, less appreciated cautionary point concerns epistasis (i.e., non-additive
85 interactions among mutations). Epistasis is commonly observed among beneficial
86 mutations, reflecting the many non-linearities in the mapping from genotype to fitness
87 that can arise at structural, metabolic, and regulatory levels^{38–41}. Of particular
88 evolutionary relevance is strong epistasis, which can alter not only the magnitude but
89 also the direction of fitness effects when mutations are combined (i.e., sign epistasis)⁴².
90 Strong epistasis may pose two challenges to attempts to exploit trade-offs for applied
91 purposes. First, since new mutations may interact idiosyncratically with previous ones,
92 there is no guarantee that any trade-offs observed in a “typical” mutant will persist
93 further along an adaptive pathway, making snapshot measurements of collateral
94 sensitivities unreliable²⁸. Second, since strong epistasis creates incompatibilities among
95 entire sets of mutations, mutational paths become contingent on which mutations
96 happen to occur first⁴³. As a consequence, strong epistasis may lead to rapid divergence
97 across multiple traits of lineages even within the same population, thus decreasing the
98 likelihood that a “typical” mutant accurately represents how populations behave in
99 secondary environments.

100

101 To explore how rapidly epistasis can drive phenotypic divergence both among and
102 within mutational paths, here we turned to the highly tractable and relevant case study
103 of enzyme-mediated antibiotic resistance. Antibiotic resistance enzymes are ideal model
104 systems for several reasons. First, they are major actors underpinning resistance in
105 clinical settings, posing significant concern due to their potential to spread rapidly
106 across bacterial species^{44,45}. Second, strong epistasis is typically pervasive among
107 beneficial mutations within enzymes, as different residues often interact with each other
108 either through direct physical contact or indirectly via altering stability and folding –
109 indeed, much of our current understanding of the causes and consequences of
110 intramolecular epistasis come from work done in the β -lactamase enzyme TEM-1^{41–43,46–}
111 ⁵⁰. Third, resistance enzymes often show substrate promiscuity across multiple
112 antibiotics within the same class, and the evolution of increased activity against non-
113 preferred substrates is a worrisome and well documented phenomenon in clinical
114 settings^{51,52}. As a result, these enzymes afford great opportunity to explore how
115 adaptation to non-preferred substrates can proceed via alternative paths with distinct
116 phenotypic profiles. Finally, for the most relevant enzymes, extensive knowledge exists
117 on the structure-function relationship of many prevalent variants, aiding with the
118 interpretation of laboratory evolution experiments.

119

120 Here we used directed evolution to assess the extent to which epistasis can drive the
121 rapid divergence across multiple traits of the clinically relevant and globally distributed
122 carbapenemase KPC-2 (*Klebsiella pneumoniae* carbapenemase-2)⁵³. This class-A serine
123 β -lactamase was originally named for its ability to hydrolyze carbapenems, last-resort
124 drugs for treating multi-drug-resistant infections⁵⁴. However, KPC-2 and its variants are
125 also active against a wide range of other β -lactam compounds, including penicillins,
126 monobactams, and cephalosporins^{55,56}. This broad-spectrum phenotype, along with its
127 rapid worldwide dissemination and high prevalence in Gram-negative bacteria, has
128 established KPC-carbapenemases as a top priority for epidemiological surveillance in
129 clinical settings^{53,57}. KPC-2 is also noted for its versatility and high evolutionary
130 potential⁵⁸; with over 200 allelic variants reported in less than three decades since it was
131 first described (bldb.eu⁵⁹). These variants have mostly diverged through point mutations,
132 although examples of small insertions and deletions also exist, and for many of the
133 variants these changes are known to result in substantial alterations of their activity
134 spectrum^{58,60}.

135

136 One of the few yet relevant compounds for which KPC-2 shows poor activity is
137 ceftazidime (CAZ)⁶¹, an important β -lactam cephalosporin commonly used in hospital
138 and community settings to treat Gram-negative and some Gram-positive bacteria⁶².
139 Leveraging this weakness, standard practice against KPC-bearing pathogens involves
140 using CAZ in combination with β -lactamase inhibitors such as avibactam (AVI)^{63–65}.
141 However, despite the fact that CAZ-AVI combination has been introduced only recently
142 (in 2015 in the USA and in 2016 in Europe), numerous reports have documented the
143 emergence of resistant mutants after treatment, typically carrying mutations that alter
144 the conformation of active-site loops to increase activity against CAZ^{58,60,66,67}. Inspired
145 by this natural case study, here we sought to characterize, via *in vitro* directed evolution,
146 the diversity of mutational paths that enable KPC-2 to confer increased resistance to
147 CAZ; placing especial emphasis on assessing how readily these paths can diverge not
148 only in terms of genotypes, but also at multiple phenotypic levels.

149

150

151 **RESULTS**

152

153 **Initial CAZ-resistance proceeds via mutations often seen in clinical variants**

154

155 To explore the potential of KPC-2 to evolve high activity against CAZ, we began by
156 cloning a wild-type *bla*_{KPC-2} gene into a high-copy-number plasmid in *E. coli* and then
157 used error-prone PCR to create 11 libraries with $\sim 10^3$ – 10^4 randomly generated mutants
158 (Methods). After overnight growth, we screened for CAZ-resistance phenotypes across
159 a gradient of doubling concentrations of CAZ, starting from the Minimum Inhibitory
160 Concentration (MIC) measured for the wild type (16 $\mu\text{g/ml}$) (Methods). From each
161 library, we selected two colonies from the highest concentration of CAZ that sustained
162 growth, which in this first round amounted to a 16-32 fold increase over the MIC of the
163 ancestor (256-512 $\mu\text{g/ml}$). Sanger sequencing of these initial 22 colonies revealed a total
164 of 38 single nucleotide polymorphisms in the *bla*_{KPC-2} gene (1.54 ± 0.66 per isolate,
165 mean \pm sd), of which 15 were unique (Table S1). These nucleotide mutations translated
166 into 34 amino acid substitutions (13 unique), arranged into nine different genotypes
167 (Table S1, Figure 1A).

168

169 We next sought to classify the first-round mutants according to the mutations most
170 likely driving the MIC increases. Clinical CAZ-resistant variants typically harbor
171 mutations in three mutational hotspots^{58,61}: the Ω -loop, forming the catalytic pocket
172 floor; loop 240, forming the active site's right wall; and loop 270, which undergoes
173 conformational changes upon CAZ binding. Each of our initial 22 mutants carried
174 mutations mapping to either the Ω -loop or loop 270. Consequently, we were able to
175 define four classes of mutants based on positions known to feature CAZ-resistance
176 mutations. The first class consists of mutants at position D178, located in the Ω -loop.
177 This position was the most commonly mutated among the initial 22 colonies (13/22,
178 59%, Table S1), and was present in more than half of the initial genotypes (5/9, Figure
179 1A). This prevalence may be linked to being the only position in which a single
180 mutation confers the highest observed MIC increase (D178Y, 32-fold), although
181 mutational biases may also play a role⁶⁸. Interestingly, reports show that this position is
182 also the one most commonly harboring single point mutations among CAZ-resistant
183 clinical variants⁵⁸. We also observed substitutions at other positions within the Ω -loop,
184 specifically L168 and S170 (2/22, 9% each, Table S1), and we took them as conforming
185 different classes with potentially different evolutionary prospects. The final class
186 comprises position H272, located in loop 270, the second most commonly altered
187 among our mutants (5/22, 23%, Table S1). Of note, a mutant carrying substitution
188 H272Y alone is known as KPC-3 in the literature⁶⁹, the most prevalent clinical variant
189 overall after KPC-2⁷⁰.

190

191 **Alternative mutational paths drive KPC-2 evolution towards CAZ-resistance**

192

193 Despite starting with 22 potential lines, the first round of directed evolution produced
194 only nine different genotypes, which fell into just four groups based on likely CAZ-
195 resistance mutations. Would these variants lead to markedly different evolutionary
196 outcomes? To increase the chances of exploring less common mutational paths, we
197 conducted a second round of directed evolution not only with the initial nine genotypes
198 but also with six duplicates chosen from across the four groups. In particular, we added
199 one line from each of the D178 and S170 groups (lines D5, S3, Table S2), and two lines
200 from each of the H272Y and L168 groups (H3, H4, L2 and L3, Table S2). These latter
201 groups were favored because they showed the smallest increases in MIC (16-fold),
202 potentially a hallmark of suboptimal paths⁵⁰. We subjected the resulting 15 lines to a

203 new round of error-prone mutagenesis and screening, following the same conditions as
204 in round one. The results contrasted to those seen before: while in round one we
205 observed large and relatively homogeneous changes in MIC (16- and 32-fold increase),
206 values in round two were much smaller and heterogeneous (from 0.5- to 8-fold increase,
207 in doubling increments) (Table S2). This heterogeneity suggests that the lines have
208 already begun to diverge substantially, with most still undergoing adaptation to different
209 extents (10/15), a few reaching a plateau (4/15, lines D5, H3, H4 and L1, Table S2), and
210 one line even experiencing a MIC decline, likely due to the random sampling of a
211 mutant with a slightly deleterious mutation (A200V, line D4, Table S2).

212

213 To guide our selection for the third round of directed evolution, we considered three
214 criteria. First, we included the 10 lines that kept sustaining adaptation thus far. Second,
215 we duplicated two lines with the highest MIC values to explore potential alternative
216 routes to maximum CAZ resistance (D1 and H1, with MICs of 1024 and 2048 $\mu\text{g/ml}$, a
217 64- and 128-fold increase over the wild-type, respectively). Third, we included three out
218 of the four lines that had reached a plateau in the previous round, as well as the line that
219 declined in MIC. The reason for continuing these lines was to investigate what drove
220 their apparent halt in adaptation: was it due to MIC-increasing mutations being just
221 infrequent, or rather because they were essentially unavailable? After these
222 considerations, we proceeded with a total of 16 lines for a third round of error-prone
223 mutagenesis and screening, under the same conditions as before. In stark contrast to
224 previous rounds, here the majority of lines showed no further adaptation (14 out of 16),
225 and the only two lines that did adapt exhibited only a modest 2-fold increase in MIC
226 (H2 and S1, Table S2).

227

228 Despite MIC increases mostly coming to a halt overall after round three, we decided to
229 subject a fraction of the lines to a final, fourth round of directed evolution. An obvious
230 choice for this last round were the two lines that were still adapting, even if modestly.
231 These lines were duplicated to further explore potential routes to maximum CAZ
232 resistance, although for H2 no further mutations appeared so the duplicate is not
233 included in Table S2. We also included five lines across groups that, despite having
234 already reached a plateau, displayed either some of the highest (D6, D7 and H5, Table
235 S2) or the lowest MICs increments overall (D4 and L1, Table S2). We included these
236 lines, representing plateaus of contrasting heights, to explore whether the non-

237 synonymous mutations acquired in the previous round could act as cryptic stepping-
238 stones towards the acquisition of further adaptive mutations⁷¹. In total, we subjected
239 nine lines to a fourth round of error-prone mutagenesis and screening. None of the lines
240 presented any further increase in MIC (Table S2).

241

242 Figure 1 summarizes the basic features and results of the four-round, directed evolution
243 experiment. Three major takeaways are possible. First, at least four distinct mutation
244 sites can mediate large-effect, first-step resistance mutations towards CAZ resistance
245 (Figure 1A). These sites, however, show a markedly uneven representation, with one
246 site accounting for 59% of the first-step mutants (D178), presumably because it enables
247 the highest single-step MIC increase. Second, while the proportion of synonymous
248 mutations showed no evidence of plateauing (Figure 1B), the MIC trajectories slowed
249 down almost in parallel across groups, with most lines reaching a plateau by round three
250 (Figure 1C). This contrast implies an excess of non-synonymous mutations during the
251 initial versus the later stages of plateaus, potentially indicating that plateaus are not
252 adaptively stagnant. Could it be that these non-synonymous mutations, invisible in
253 terms of MIC levels, are mediating the cryptic adaptive evolution of other fitness-
254 related traits?

255

256 The third takeaway is that first-step mutations directed adaptation through distinct,
257 mostly non-overlapping sets of adaptive mutations, indicating that strong epistatic
258 incompatibilities are pervasive⁴³. Indeed, only 6 out of the 35 sites featuring mutations
259 were hit in more than one group (17%, L25, P93, E167, S196, V239, and H272; Figure
260 1D), with only one site hit in three groups (P93), and none in all. However, this stark
261 genetic divergence across groups translates into just moderate levels of divergence in
262 CAZ-resistance (Figure 1C). Indeed, only groups L168 and S170 show statistically
263 significant differences in endpoint MIC levels ($P = 0.024$, Student's t-test on log₂
264 transformed values). In other words, the different groups may be exploiting alternative
265 solutions at the molecular level, and yet these solutions converge to similar values of the
266 major fitness-related trait in the selective conditions. While this convergence could be
267 interpreted in terms of global physio-chemical limits to enzymatic activity, it is still
268 possible that the different genotypes behave differently across secondary traits that
269 matter to fitness in other environments. Could the high degree of genetic divergence
270 translate into markedly different properties beyond the original selective conditions?

271 **Collateral sensitivities rapidly diverge during CAZ-resistance evolution**

272

273 We next sought to assess the extent to which the different lines adapted to CAZ may
274 show cross-resistance or cross-sensitivity to other beta-lactam antibiotics, as cycling
275 among beta-lactams has been proposed as a convenient intervention to curb resistance
276 evolution⁷²⁻⁷⁴. To this end, we chose three compounds commonly used to treat Gram-
277 negative infections, with different degrees of chemical similarity to CAZ⁷⁵. These
278 include cefotaxime (CTX), which belongs to the same subclass as CAZ (3rd generation
279 cephalosporins); cefoxitin (FOX), a 2nd generation cephalosporin; and imipenem (IMI),
280 an important representative of the carbapenem class. Figure 2A reveals a general trend
281 for CAZ-adapted lines to become more susceptible than the ancestor to the other
282 antibiotics, although the fraction of lines showing this increased susceptibility varies
283 considerably (92.9% for IMI, 78.6% for FOX, and 57.1% for CTX). Another relevant
284 point is that MIC trajectories for the other antibiotics show less clear plateaus than those
285 seen in CAZ, with frequent fluctuations occurring at rounds where CAZ MIC values
286 have already stabilized. This observation provides further evidence that the non-
287 synonymous mutations detected in plateaus have some functional relevance: these
288 changes are probably driving the adaptive evolution of other fitness components and,
289 while not affecting CAZ MIC values, manifest in the MIC values for other drugs.

290

291 To further investigate the observed tendency towards collateral sensitivity, we examined
292 how the overall magnitude of collateral effects changed through rounds. Since most
293 high-impact CAZ resistance mutations were incorporated early on, it is reasonable to
294 expect the most pronounced collateral effects to occur early as well^{76,77}. Figure 2B
295 confirms this expectation, but also shows that while these effects tend to decay over
296 time (right top panel), they do at varying rates. Indeed, there is a significant negative
297 correlation for strong collateral effects occurring mostly at the beginning of the
298 experiments for CTX and IMI (Pearson's $R = -0.68$ and -0.42 , respectively; $P < 0.01$
299 for both), but not for FOX ($R = -0.27$; $P = 0.068$). We then focused on collateral effects
300 at the level of each individual mutational step, looking for correlations between the
301 change in MIC for CAZ versus the other antibiotics. The remaining panels in Figure 2B
302 reveal that only individual collateral effects in IMI exhibit a significant correspondence
303 with the primary effects seen in CAZ ($R = -0.74$; $P < 10^{-7}$), whereas the correspondence
304 for CTX and especially FOX is virtually absent ($R = 0.21$ and $R = 0.05$; $P = 0.180$ and

305 0.741, respectively). The discrepancy in statistical significance observed for CAZ
306 between absolute, global effects and actual, individual ones likely reflects the
307 substantial degree of divergence that lines experienced in this phenotype, with some
308 developing cross-sensitivity and others cross-resistance (Figure 2A).

309

310 We next sought to assess the extent to which epistasis can cause heterogeneity in
311 collateral sensitivity profiles. This heterogeneity can manifest in two dimensions:
312 among lines, and over time along each line. First, we focused on heterogeneities among
313 endpoint isolates, examining differences both among and within each group. Figure 2C
314 shows that endpoint sensitivity profiles vary markedly among groups, primarily due to
315 the tendency of lines in the H272 group to exhibit MIC values that consistently rank
316 highest across antibiotics ($P < 0.002$, two-sample Wilcoxon test on ranked data).
317 However, we note that a few lines deviate from the typical profile of their group (H3
318 and, to a lesser extent, S2). This deviation illustrates that, even when initial mutations
319 markedly determine the properties of subsequent adaptive steps, some secondary
320 mutations can cause pathways to branch away from the overall profile of the main route.
321 Interestingly, in both H3 and S2, these deviations involve mutations that do not affect
322 CAZ MIC values (Table S2), illustrating how cryptic changes during plateaus can have
323 unexpected consequences in phenotypic profiles.

324

325 Finally, and in line with the last point, we sought to quantify how much divergence
326 typically happens over time along a single mutational path. To this end, for each line
327 and secondary antibiotic combination, we classified first-step mutants as cross-resistant,
328 neutral, or cross-sensitive compared to the ancestor; and then assessed how this
329 classification changed for endpoint mutants. Figure 2D shows the extent and direction
330 to which endpoint isolates deviate from the collateral sensitivity profile of the first-step
331 mutant of their respective lines. We found that the most common paths were those along
332 which sensitivities changed classification (33%, 14/42), followed by paths along which
333 effects were aggravated (31%, 13/42), paths with no change (19%, 8/42), and paths
334 along which effects were mitigated (16.7%, 7/42). Notably, all paths with mitigating
335 effects involved cross-sensitive first-step mutants.

336

337

338

339 **Molecular traits rapidly diverge during CAZ-resistance evolution**

340

341 Thus far, we have interpreted our results in terms of MICs, which are macroscopic
342 readouts that arise from the aggregate contributions of multiple molecular-level traits.
343 Among these, the traits typically considered to shape MIC the most are those directly
344 affecting how efficiently an enzyme converts substrate into product (i.e., the “catalytic
345 efficiency”, the ratio of the turnover number over the substrate affinity, k_{cat}/K_m)^{49,78}.
346 However, recent work revealed a major role for the multiple traits that affect the
347 different aspects of the beta-lactamase’s life cycle, including its exit from the ribosome
348 as a precursor polypeptide, translocation across the inner membrane, folding once in the
349 periplasm, and their eventual degradation by proteases^{79–83}. The intuition for this
350 importance is that, regardless of how efficient the beta-lactamase may be, these factors
351 ultimately determine effective reaction rates by controlling the steady-state abundance
352 of functional enzymes in their natural workplace⁸³.

353

354 Although the specific molecular traits altered are often difficult to pinpoint, an
355 aggregate readout of all these traits can be obtained by directly measuring enzyme
356 abundance stability in the periplasm^{79,81}. Indeed, these periplasmic stability
357 measurements have successfully helped explain MIC differences among closely related
358 enzyme variants⁸³, and most revealingly, among the same enzyme across different
359 hosts⁸². Moreover, beyond MICs, these traits largely control fitness costs in the absence
360 of drugs, as alterations in any stage of the enzyme's life cycle can interfere with other
361 cellular processes, such as saturating the translation, membrane translocation, and
362 folding machinery, or triggering stress responses due to the aggregation of misfolded
363 proteins^{84,85}. This impact on fitness in the absence of strong selective pressures for
364 resistance is expected to be crucial for the long-term success of enzyme variants under
365 ever-changing conditions^{82–84}.

366

367 To gain insights into how epistasis may have driven divergence in molecular-level traits
368 during CAZ adaptation, we estimated the catalytic efficiency (k_{cat}/K_m), periplasmic
369 abundance stability and growth rates of a fraction of the lines. The results are
370 summarized in Figure 3. We first examined the catalytic efficiency of purified extracts
371 for several variants (Methods), including all steps in the line that achieved the largest
372 MIC increase (H2), an alternative endpoint isolate deviating from this line (H1), and

373 three high MIC representative isolates from across the other groups (D1, S1 and L2).
374 Figure 3A shows variations in catalytic efficiency spanning more than three orders of
375 magnitude, including one line in which almost no increase relative to the ancestor was
376 detected (D178Y). These observations contrast to the fact that CAZ MICs increased
377 substantially in all lines, and that in endpoint isolates MIC values vary less than 10-fold
378 (512 – 4096 µg/ml, Table S1). Similar results were obtained using hydrolysis rate
379 estimates from a colorimetric assay, an alternative method for assessing pure enzymatic
380 activity⁶⁰ (Figure SX) (Methods). Finally, we ask if at least at the level of each
381 individual step, the effects on MIC and catalytic efficiency show any degree of
382 correlation (Figure 3A, bottom panel). In line with previous studies^{49,81,83}, no statistically
383 significant correlation was detected ($R = 0.01$; $P = 0.98$).

384

385 We next examined the stability of enzyme abundance levels in the periplasm (i.e., in-
386 cell kinetic stability, dubbed this way to differentiate it from standard, *in vitro* thermal
387 stability⁸⁶). To this end, we added a hemagglutinin tag to wild-type KPC-2 and several
388 evolved variants, and used Western blot to quantify the change in their relative
389 abundance in the periplasm over time (Figures SX and SY) (Methods). For this study,
390 we chose two high-MIC lines from each group, including all the individual mutants
391 from each line. Figure 3B reveals a common pattern in which periplasmic stability
392 systematically drops during the first steps of adaptation, later recovering to different
393 extents in all lines, except from the D178-derived ones. In addition, while here again the
394 values for the endpoint isolates vary widely (four-orders of magnitude), analyses at the
395 level of each individual mutation step show a clear negative correlation between the
396 effects on MIC and on periplasmic stability ($R = -0.7$; $P = 0.08$) (Figure 3B, bottom
397 panel). Interestingly, the largest individual effect on stability in this panel corresponds to
398 a mutation that does not change MIC (L1, E167G, Table S1), reinforcing the idea that
399 adaptive evolution did not stop once populations reached the MIC plateaus. Further
400 support for this idea comes from the observation that endpoint isolates from S170P-
401 derived lines exhibited an altered molecular weight at later incubation times (Figure
402 SX). Proteomics confirmed the presence of the enzyme, suggesting the possibility of
403 structural or post-translational modifications that may enhance stability by facilitating
404 binding to other components of the periplasm.

405

406 We then measured growth rates as a proxy to capture any collateral fitness costs
407 resulting from the evolution towards CAZ resistance^{84,85}. We conducted the assays at
408 sub-inhibitory concentrations of CAZ not only to be able to include the ancestor, but
409 also to assess the severity of the potential costs under conditions where there is still a
410 low, yet consequential, demand for the enzymes to perform their physiological
411 function⁸⁷. We first estimated growth rates for the same two high-MIC lines from each
412 group used in the periplasmic stability study, including all individual mutants along
413 lines. Figure 3C shows a general trend whereby growth rates become larger with each
414 round of evolution, which is expected simply due the higher protective capacity of the
415 evolved enzymes. However, endpoint growth rates exhibit variations that do not align
416 with MIC difference. This is best exemplified by line L1, which displays the highest
417 growth rate despite having the lowest MIC (256 µg/ml, Table S1). Similarly, S170-
418 derived lines show the lowest growth rates even though they have some of the highest
419 MIC values (1024 and 2048 µg/ml, S1 and S2, respectively, Table S1). At the level of
420 each individual mutation step, we found a significant positive correlation between the
421 effects on MIC and on growth rate ($R = 0.77$; $P = 0.02$) (Figure 3C, bottom panel).
422 Lastly, it is worth noting line L2, which undergoes a large drop in growth rates in round
423 three, followed by a full recovery in the next round. This recovery involves a mutation
424 that does not alter MIC (E167D, Table S1), again pointing at the non-static nature of
425 plateaus.

426

427 Finally, we wanted to characterize the patterns of change occurring specifically during
428 the plateaus. We first examined periplasmic stability, looking at the direction of change
429 between consecutive mutants within each plateau. Figure 3D shows that periplasmic
430 stability increases during plateaus in all lines, except for the D178-derived ones (6/8,
431 Wilcoxon Rank Sum test, $P > 0.05$ in all cases). We then turned our attention to growth
432 rates, for which we extended growth curve measurements to all mutants in plateaus
433 across lineages (Figure SZ). Figure 3D reveals that, despite MIC stasis, a majority of
434 lines exhibit a significant increase in growth rate (8/15), while only three lines show
435 significant decreases (Wilcoxon Rank Sum test, $P > 0.05$ in all cases). Overall, these
436 results point at the idea that adaptive evolution continues across multiple molecular
437 traits even after CAZ-resistance levels stabilize. Moreover, a glance at the location and
438 predicted effects of mutations over rounds provides further support for this notion
439 (Figure 4). Initial mutations involve disruptive substitutions near the active site, likely

440 reshaping its physical and chemical properties to enhance enzymatic activity. Later
441 mutations tend to occur farther away and have milder effects, possibly compensating for
442 the stability loss and collateral fitness costs incurred by the initial activity-enhancing
443 mutations. Similar patterns of mutations falling progressively away from the active
444 center have been observed in directed evolution experiments with other proteins^{88,89}.

445 **DISCUSSION**

446

447 In this study, we sought to assess the extent to which strong epistasis can lead lineages
448 to diverge not only at the genotypic level, but also across other traits that may be
449 relevant in alternative conditions. To this end, we characterized the diversity of
450 mutational paths through which the globally distributed KPC-2 carbapenemase can
451 evolve increased activity towards a poor substrate, the beta-lactam antibiotic CAZ. Our
452 results show that KPC-2 can readily adapt through different mutational paths with
453 markedly distinct phenotype properties, including differences in catalytic efficiency,
454 periplasmic stability, collateral fitness costs, and susceptibility to other antibiotics. The
455 speed and degree of divergence observed, both among and within lines, are remarkable
456 considering that we conducted the experiments under identical and well-controlled
457 laboratory conditions; and bear implications for designing strategies to prevent
458 antibiotic resistance, and for understanding natural evolution in complex environments.

459

460 Can we rationalize the molecular basis for why the initial CAZ-resistance mutations
461 lead to such divergent outcomes? We classified the lines into four groups based on the
462 positions altered in the initial CAZ-resistance mutations. These positions, however, vary
463 in how frequently they are altered among all natural KPC-2 variants: at present, position
464 H272 retrieves the most hits (47%) in the BetaLactamase DataBase (<http://blddb.eu>)⁵⁹,
465 followed by D178 (26%), L168 (15%), and S170 (9%). Reflecting this unequal
466 prevalence, detailed studies on the molecular mechanisms of resistance are available
467 only for positions D178 and H272. Substitutions N and Y at D178 increase Ω loop
468 flexibility, facilitating the rapid entry of CAZ into the active site^{90,91}. However, this
469 flexibility reduces hydrolysis rate due to the displacement of essential hydrolytic
470 residues. It is not surprising, therefore, the reductions in both stability and hydrolytic
471 efficiency that we observed (Figure 3A-B, Figure SX). Interestingly, increased
472 resistance in D178 mutants is attributed to a mechanism referred to as a “kinetic trap”,
473 whereby the swift entry of CAZ into the reaction pocket reduces to safe levels the
474 amount of free molecules available for inhibiting penicillin-binding proteins⁹¹. In stark
475 contrast, substitution Y at H272 enhances hydrogen bonding with CAZ's oxyimino side
476 chain, directly increasing the hydrolysis rate⁶⁰. Additionally, H272Y is thought to aid
477 CAZ entry into the active site without altering the Ω loop's flexibility, which is achieved
478 by the repositioning of the surrounding 240 and 270 loops⁹². So to sum up, these well-

479 studied positions represent fundamentally different mechanisms for enhancing beta-
480 lactamase activity; being therefore reasonable to expect that they may give rise to
481 distinct phenotypic profiles.

482

483 Among the implications of our findings, the most immediate pertains to current efforts
484 to exploit collateral sensitivities in the fight against antibiotic resistance⁹³. Our work
485 demonstrates that widespread strong epistasis can drive the rapid and substantial
486 divergence of resistance profiles across different antibiotics, even in parallel replicates
487 evolving under the same conditions. Moreover, this divergence can occur not only
488 among lines initiated from distinct mutations, but also along the sequential steps that
489 comprise each line. These observations reveal extra challenges to the endeavor of
490 anticipating evolutionary responses to antibiotics^{28–33,35}. For instance, resistance trade-
491 offs may markedly differ among seemingly similar populations, due to being dominated
492 by the descendants of different mutants that happened to be the first to occur.
493 Furthermore, heterogeneities in resistance are also expected among individuals within a
494 single population, provided its size is sufficiently large. To further complicate matters,
495 the observed trade-offs may not even be stable over subsequent steps of adaptive
496 evolution. These phenomena call into question the reliability of snapshot
497 characterizations of “typical” mutants as indicators of populations’ responses to future
498 treatments²⁸. Thus, this work adds to the cautionary evidence suggesting that collateral
499 sensitivity strategies, while promising, need to be carefully tailored to take into account
500 the many factors influencing evolutionary repeatability^{28–33,35}. This body of observations
501 calls for a careful and thorough characterization of the spectrum of resistance mutations,
502 within the limits of technical feasibility, if we are to ensure the best success of these
503 strategies.

504

505 Our findings also bear implications for understanding the natural evolution of antibiotic
506 resistance enzymes, as well as the distribution patterns of variants across regions and
507 species. Bacterial populations, especially in clinical pathogens, are regularly exposed to
508 multiple antibiotics with concentrations that can change unpredictably over space and
509 time^{87,94–96}. Since phenotypic profiles are strongly determined by the chance occurrence
510 of initial mutations, which variants will prevail in a particular setting will be heavily
511 contingent upon the specific order and intensity of the antibiotic pressures encountered
512 by the population. Small population sizes or transmission bottlenecks will further

513 amplify this historical contingency, simply due to the stochastic sampling of different
514 variants with potentially distinct properties in other environments^{28,50}.

515

516 Moreover, besides differences in antibiotic resistance profiles, we have shown that
517 strong epistasis can readily lead to variants with substantial differences in periplasmic
518 abundance and collateral fitness costs, traits that presumably reflect how enzymes
519 interact with various cellular components throughout their life cycle⁸²⁻⁸⁵. These
520 differences are expected to play a role in the scenario described above, favoring the
521 selection of the least costly variants when antibiotics are at very low concentrations or
522 even absent^{84,85}. On the long run, these differences are probably critical for the
523 evolutionary success of variants during their journey across multiple hosts^{82,83,97}. Indeed,
524 while we have presented evidence that variants interact differently with the cellular
525 components of just one host, these differences may be amplified or mitigated in other
526 hosts with their own idiosyncrasies in their cellular components. Thus, strong epistasis
527 might fuel the rapid association of certain variants with specific hosts, a particularly
528 intriguing possibility given how important horizontal gene transfer is in the
529 dissemination of antibiotic resistance genes^{44,45}.

530

531 Lastly, it is pertinent to ask whether the strong degree of epistasis-driven phenotypic
532 divergence observed here with KPC-2 is peculiar to this enzyme, or if, conversely,
533 similar patterns can be expected in other model systems. KPC-2 has been previously
534 hypothesized to be more evolvable than other class A beta-lactamases because its
535 thermal stability is significantly higher than other model enzymes like TEM and CTX-
536 M (66.5°C versus 51.5°C and 51°C, respectively)⁶⁰. High thermal stability enables
537 enzymes to accumulate destabilizing beneficial mutations while maintaining proper
538 folding and functionality, a property shown to boost evolvability in experiments with
539 different systems^{98,99}. Thus, compared to other class A beta-lactamases, an intriguing and
540 testable possibility is that KPC-2's enhanced stability may facilitate the acquisition of
541 first-step mutations with a broader and more diverse range of molecular mechanisms for
542 increasing enzymatic activity, being thus more prone to lead to divergent outcomes
543 across multiple traits.

544

545 Beyond single proteins, it remains to be determined whether epistasis-driven phenotypic
546 divergence is a general phenomenon in bacterial rapid adaptation to novel conditions.

547 For instance, strong epistasis is common in the well-known Long Term Evolution
548 Experiment with *E. coli*, in which 12 populations have been adapting to a low-glucose
549 environment for over 75,000 generations¹⁰⁰. However, the level of divergence across
550 secondary environments that lineages experienced is relatively moderate, and it
551 typically took thousands of generations to become manifest^{101,102}. One possible
552 explanation for this difference in breadth and pace is that in the LTEE selection
553 pressures are milder. In addition, the functional target size for adaptation in the LTEE
554 involves multiple genes, and therefore adaptation is not entirely dominated by the
555 biophysical details of a single protein. Supporting this interpretation, a similar
556 experiment that adapted 114 lines of *E. coli* to a nearly lethal temperature found much
557 higher levels of divergence within just 2000 generations¹⁰³. In this study, lines diverged
558 along two epistatically incompatible mutational paths involving strongly adaptive
559 mutations in key hubs of gene expression regulation. Notably, one path led to collateral
560 resistance against rifampicin, an antibiotic never present in the medium¹⁰⁴, providing a
561 clear illustration of epistasis-driven divergence during genome-wide evolution. Further
562 research is needed to clarify the factors that determine the degree to which strong
563 epistasis can produce markedly different outcomes across environments.

564 **METHODS**

565

566 **Bacterial strains and plasmids**

567 *E. coli* TOP10 strain (Invitrogen, Massachusetts, USA) and the pUC-derived, high-copy
568 number pCR-blunt II-TOPO vector (Invitrogen), carrying a kanamycin resistance gene,
569 were used in the random mutagenesis experiments, susceptibility testing, growth curves
570 and periplasm extract collection. *E. coli* Origami 2(DE3) strain (Novagen, Fontenay-
571 sous-Bois, France) and pET-28a plasmid (Novogen) were used for protein expression
572 and purification.

573

574 **Random mutagenesis**

575 Wild-type *bla_{KPC-2}* gene was amplified from a reference strain obtained from Dr. Naas
576 laboratory¹⁰⁵ (env-KPC-2_Fw: 5' TTC AAA CAA GGA ATA TCG TTG ATG TCA CTG
577 TAT CGC CGT CT 3', env-KPC-2_Rv: 5' AAT AGA TGA TTT TCA GAG CCT TAC
578 TGC CCG TTG ACG CCC A 3'), cloned into the pCR-blunt II-TOPO vector
579 (Invitrogen), and expressed in *E. coli* TOP10 (Invitrogen). Random mutagenesis was
580 performed using the GeneMorph II EZClone Domain Mutagenesis Kit (Agilent)
581 according to the manufacturer's instructions. The *bla_{KPC-2}* gene was amplified with a
582 similar pair of primers as above but sparing the start of the open reading frame (PRE-
583 KPC-2_Fw: 5' TTC AAA CAA GGA ATA TCG TTG 3', PRE-KPC-2_Rv: 5' AAT AGA
584 TGA TTT TCA GAG CC 3') and using Mutazyme II DNA polymerase, which according
585 to the manufacturer, generates a low-bias mutation spectrum with similar mutation rates
586 at A's/T's and G's/C's. To achieve a low mutation rate (0–4.5 mutations/kb), 500 ng of
587 target DNA and 30 PCR cycles were used. The resulting variant library was purified
588 using the GeneJET PCR Purification Kit (ThermoFisher). A second PCR, using the
589 mutant library as megaprimers and the pCR-blunt II-TOPO (*bla_{KPC-2}*) plasmid as the
590 template, was performed. In the next step, the template was digested with Dpn1
591 (ThermoFisher), and the PCR product was transformed into *E. coli* TOP10.
592 Transformants were incubated overnight at 37°C in LB broth supplemented with 50
593 µg/ml kanamycin to ensure plasmid retention. Mutant selection was performed in
594 Mueller Hinton (MH) broth (ThermoFisher). Four tubes containing 20 mL of MH broth
595 with increasing concentrations of ceftazidime (CAZ) (ThermoFisher) were used. The
596 first concentration corresponded to the midpoint of the minimal inhibitory concentration
597 (MIC) conferred by KPC-2, and subsequent concentrations were doubled. Inocula were

598 adjusted to 5×10^5 CFU/mL, and cultures were incubated overnight at 37°C. The tube
599 with the highest CAZ concentration showing visible growth was plated on LB agar with
600 50 µg/mL kanamycin. For the first mutagenesis round, two randomly selected colonies
601 were sent for Sanger sequencing using M13 primers (M13_Fw 5' GTA AAA CGA CGG
602 CCA G 3', M13_Rv 5' CAG GAA ACA GCT ATG AC 3'), which were also used in
603 subsequent rounds.

604

605 **Susceptibility testing**

606 MIC values for *E. coli* TOP10 strains carrying KPC mutants were determined using the
607 broth microdilution method following EUCAST guidelines. In brief, 96-well plates
608 were used with a final volume of 200 µL per well. In the first well, 100 µL of MH broth
609 and antibiotic stock (final concentration < 25% of 100 µL) were added to achieve the
610 highest concentration tested. Serial two-fold dilutions of the antibiotic were then
611 prepared. Finally, 100 µL of inoculum (1×10^6 CFU/mL) from an overnight culture was
612 added. MIC values were assessed after 18 hours of incubation at 37°C.

613

614 **Growth Curves**

615 A 300 µL volume of LB broth containing 8 µg/mL ceftazidime was inoculated with *E.*
616 *coli* TOP10 expressing KPC-2 and its variants and placed in 100-well honeycomb
617 plates. Incubation was carried out at 37°C for 20 hours using the Bioscreen C Pro
618 Microbiological Growth Analyser (Labsystems, Helsinki, Finland), with OD600 values
619 measured every 10 minutes. Five replicates were used per variant. Growth rate values
620 were determined from the maximum slope of the natural logarithm of optical densities
621 over time.

622

623 **Protein purification**

624 The *bla*_{KPC-2} gene and variants' fragments corresponding to the mature β-lactamase were
625 cloned into the expression vector pET28a, using the PCR generated fragment with
626 primers NdeI-KPC-2-sps_Fw (5' CAT ATG CTG ACC AAC CTC GTC GCG GA 3'),
627 BlnI-KPC-2_Rv (5' GCT CAG CTT ACT GCC CGT TGA CGC CCA AT 3') and the
628 NEBuilder® HiFi DNA Assembly Cloning Kit (New England BioLabs®Inc, United
629 Kingdom), following the manufacturer's instructions. Recombinant plasmids were
630 transformed into chemocompetent *E. coli* Origami 2(DE3) and the transformant were
631 selected in LB agar plates supplemented with 50 µg/ml kanamycin. An O/N culture of

632 *E. coli* Origami 2(DE3) harboring pET28a(*bla*_{KPC-2} gene or *bla*_{KPC}-mutant genes) was
633 used to inoculate 2 L of LB broth containing 50 µg/L kanamycin. Bacteria were cultured
634 at 37°C until reaching an OD of 0.6 at 600 nm. Expression of KPC-2 and variants was
635 induced O/N at 25°C with 0.1 mM IPTG. The *bla*_{KPC-2} gene and its mutants were
636 purified in one step pseudo-affinity chromatography using a NTA-Nickel column
637 (Cytiva, Freiburg, Germany). Protein purity was estimated by SDS-PAGE, pure
638 fractions were pooled and dialyzed against 20mM Hepes SO4K2 50 mM buffer (pH=7)
639 and concentrated by using Vivaspin® columns (Cytiva, Freiburg, Germany). Protein
640 concentration was determined by Bradford Protein assay (Bio-Rad, Marnes-La-
641 Coquette, France).

642

643 **Steady state parameters**

644 Kinetic parameters of purified KPC-2 and its mutants were determined at 25°C in 100
645 mM sodium phosphate buffer (pH=7). The k_{cat} and K_m values were determined by
646 analyzing hydrolysis of β-lactams under initial-rate conditions with an ULTROSPEC
647 2000 model UV spectrophotometer (GE Healthcare). Kinetic parameters were
648 calculated using the non-linear regression of the Michaelis-Menten equation $v =$
649 $k_{cat}[S]/(K_m+[S])$. The β-lactams were purchased from Sigma-Aldrich (Saint-Quentin-
650 Fallavier, France). The velocity of ceftazidime hydrolysis could not be saturated by
651 measurable concentrations due to a high K_m . Thus, the second order rate constant at
652 steady-state, k_{cat}/K_m , was determined by fitting the progress curves to the equation $v =$
653 $k_{cat}/K_m[E][S]$, where $[S] \ll K_m$ ⁶⁰.

654

655 **Periplasmic stability**

656 A C-terminal hemagglutinin tag was added to the proteins expressed by *E. coli* TOP10
657 strains harboring pCR-blunt II-TOPO (*bla*_{KPC-2} gene and variants) by using PRE-KPC-
658 2_Fw and KPC-HA-STOP_Rv primers (5' TTC AAA CAA GGA ATA TCG TTG 3', 5'
659 TTA TGC ATA ATC CGG AAC ATC ATA CGG ATA CTG CCC GTT GAC GCC C 3').
660 Extraction of periplasmic proteins was adapted from⁸⁶. *E. coli* TOP10 pCR-blunt II-
661 TOPO strains (*bla*_{KPC-2} gene and variants) were incubated in LB broth supplemented
662 with 50 µg/L kanamycin until reaching a OD₆₀₀ value of 0.30, which was taken as T₀.
663 Two ml aliquots of the cultures were then taken at 1, 2, 5 and 10 h from T₀. The aliquots
664 were pelleted, washed with 20 mM Tris-HCl (pH 8.5) and 150 mM NaCl. To normalize
665 the number of cells, the volume according to $V = (2 \text{ ml} \times 0.064 \text{ OD}_{600})/\text{OD}_{600}$ was

666 resuspended in 1ml of 20 mM Tris-HCl (pH 8.5), 0.1 mM EDTA, 20% w/v sucrose, 1
667 mg/ml lysozyme (Sigma-Aldrich, protein $\geq 90\%$), 0.5 mM phenylmethylsulfonyl
668 fluoride (PMSF), incubated with gentle agitation at 4 °C for 30 min. After centrifugation
669 at 15000 g for 2 min at 4°C, the supernatant containing the periplasmic fraction was
670 collected and store at 4°C. Levels of C-terminal HA tagged proteins were determined in
671 the periplasmic fraction, by SDS–PAGE, transferred to iBlot™ 3 Transfer Stacks,
672 nitrocellulose (ThermoFisher), followed by western blot with rat anti-HA high affinity
673 monoclonal antibodies (Sigma-aldrich, Missouri, United States) at 1:1000 dilution for
674 KPC-2 and its variants. Goat anti-rat immunoglobulin Horseradish Peroxidase (HRP)
675 (Invitrogen), at 1:10000 dilution, were used as secondary antibodies. SuperSignal West
676 Pico PLUS Chemoluminescent substrate (ThermoFisher) was use for detection. Protein
677 band intensities were quantified with ImageJ. The Precision Plus Protein Standards
678 Kaleidoscope (BioRad, California, United States) provided molecular weight standards.
679

680 **Proteomic analysis**

681 Protein identification and characterization by Liquid Chromatography with tandem
682 mass spectrometry (LC-MS/MS) were carried out at the CBM-CSIC protein chemistry
683 facility (Madrid, Spain). Briefly, in-gel digestion was performed after SDS-PAGE of the
684 periplasmic extract. Gel bands of the target molecular weight were dried, destained in
685 acetonitrile (1:1), reduced with 10 mM DTT at 56°C for 1 hour, and alkylated with 10
686 mM iodoacetamide for 30 minutes at room temperature in darkness. The proteins were
687 then digested in situ with sequencing-grade trypsin (Promega, Madison, WI), following
688 the method described by Pérez *et al.*¹⁰⁶. The gel pieces were dehydrated with
689 acetonitrile, then dried in a speedvac. They were rehydrated in 100 mM Tris-HCl (pH
690 8), 10 mM CaCl₂ with 12.5 ng/μl trypsin for 1 hour on ice. After removing the digestion
691 buffer, the gels were incubated with fresh 100 mM Tris-HCl (pH 8), 10 mM CaCl₂ at
692 37°C for 12 hours. Digestion was stopped by adding 1% TFA. The supernatants were
693 dried and desalted using ZipTip C18 pipette tips (Millipore) before mass spectrometric
694 analysis.

695

696 The desalted protein digest was resuspended in 10 μl of 0.1% formic acid and analyzed
697 by Reverse Phase Liquid Chromatography coupled with Tandem Mass Spectrometry
698 (RP-LC-MS/MS) using an Easy-nLC II system and an LTQ-Orbitrap-Velos-Pro mass
699 spectrometer (Thermo Scientific). Peptides were concentrated on-line with a 0.1 mm ×

700 20 mm C18 RP precolumn (Thermo Scientific) and separated using a 0.075 mm × 250
701 mm bioZen 2.6 μm Peptide XB-C18 RP column (Phenomenex) at 0.25 μl/min. A 60-
702 minute dual gradient was applied: 5–25% solvent B for 45 min, 25–40% solvent B for
703 45 min, 40–100% solvent B for 2 min, and 100% solvent B for 18 min (Solvent A:
704 0.1% formic acid in water; Solvent B: 0.1% formic acid, 80% acetonitrile in water). ESI
705 ionization was performed with a Nano-bore Stainless Steel emitter (Proxeon) at 2.1 kV,
706 with S-Lens set at 60%. The Orbitrap resolution was 30,000. Survey scans (400–1600
707 amu) were followed by twenty data-dependent MS/MS scans (Top 20), with an isolation
708 width of 2 u, normalized collision energy of 35%, and dynamic exclusion for 60
709 seconds. Charge-state screening rejected unassigned and singly charged ions.

710

711 Peptide identification from raw data was carried out using PEAKS Studio vXPro/ v11.5
712 search engine (Bioinformatics Solutions Inc., Waterloo, Ontario, Canada). Database
713 search was performed against uniprot-escherichia-coli.fasta (4403 entries; UniProt
714 release 05/2023). (decoy-fusion database). The sequence of the KPC-2-HA protein has
715 been included in this database. The following constraints were used for the searches:
716 tryptic cleavage after Arg and Lys, up to two missed cleavage sites, and tolerances of 20
717 ppm for precursor ions and 0.6 Da for MS/MS fragment ions and the searches were
718 performed allowing optional Met oxidation and Cys carbamidomethylation. False
719 discovery rates (FDR) for peptide spectrum matches (PSM) and for protein was limited
720 to 0.01. Only those proteins with at least two unique peptides being discovered from
721 LC/MS/MS analyses were considered reliably identified.

722

723 **Statistical analyses and data visualization**

724

725 All statistical analyses were conducted using the R programming language (version
726 3.6.3), utilizing built-in functions and available packages. Pearson correlation
727 coefficients, linear regression, Student's t-Test, Wilcoxon Rank Sum test, and pairwise
728 Wilcoxon signed-rank tests were performed with the functions *cor()*, *lm()*, *t.test()*,
729 *wilcox.test()*, and *pairwise.wilcox.test()*, respectively. Data visualization for Figures 1C
730 and 2C used the “pheatmap” and “rgl” libraries, respectively. Figure 3A was generated
731 using ChimeraX.

732

733 **ETHICS**

734

735 This work did not require ethical approval from a human subject or animal welfare
736 committee.

737

738 **DATA ACCESSIBILITY**

739

740 All data necessary to replicate the research findings is available, either at public data
741 repositories (TBD) or in the electronic supplementary material (TBD).

742

743 **AUTHORS' CONTRIBUTIONS**

744

745 **LD:** Conceptualization (equal); methodology (lead); investigation (lead); formal
746 analysis (supporting); writing – original draft (supporting); writing – review and editing
747 (equal), funding acquisition (supporting), supervision (supporting). **IN:** investigation
748 (supporting); writing – review and editing (supporting). **AC:** Conceptualization (equal);
749 formal analysis (lead); writing – original draft (lead); writing – review and editing
750 (equal), funding acquisition (lead), supervision (lead).

751

752 **CONFLICT OF INTEREST DECLARATION**

753

754 The authors declare no competing interests.

755

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768 REFERENCES

1. Smith, J. M. & Haigh, J. The hitch-hiking effect of a favourable gene. *Genet. Res.* **23**, 23–35 (1974).
2. Berry, A. J., Ajioka, J. W. & Kreitman, M. Lack of polymorphism on the Drosophila fourth chromosome resulting from selection. *Genetics* **129**, 1111–1117 (1991).
3. Stephan, W. Selective Sweeps. *Genetics* **211**, 5–13 (2019).
4. Atwood, K. C., Schneider, L. K. & Ryan, F. J. Periodic selection in Escherichia coli. *Proc. Natl. Acad. Sci. U. S. A.* **37**, 146–155 (1951).
5. Hermisson, J. & Pennings, P. S. Soft sweeps: molecular population genetics of adaptation from standing genetic variation. *Genetics* **169**, 2335–2352 (2005).
6. Schenk, M. F. *et al.* Population size mediates the contribution of high-rate and large-benefit mutations to parallel evolution. *Nat. Ecol. Evol.* **6**, 439–447 (2022).
7. Good, B. H., McDonald, M. J., Barrick, J. E., Lenski, R. E. & Desai, M. M. The Dynamics of Molecular Evolution Over 60,000 Generations. *Nature* **551**, 45–50 (2017).
8. Pennings, P. S. & Hermisson, J. Soft sweeps II--molecular population genetics of adaptation from recurrent mutation or migration. *Mol. Biol. Evol.* **23**, 1076–1084 (2006).
9. Lee, M.-C. & Marx, C. J. Synchronous Waves of Failed Soft Sweeps in the Laboratory: Remarkably Rampant Clonal Interference of Alleles at a Single Locus. *Genetics* **193**, 943–952 (2013).
10. Blanquart, F., Kaltz, O., Nuismer, S. L. & Gandon, S. A practical guide to measuring local adaptation. *Ecol. Lett.* **16**, 1195–1205 (2013).
11. Sparks, M. M., Kraft, J. C., Blackstone, K. M. S., McNickle, G. G. & Christie, M. R. Large genetic divergence underpins cryptic local adaptation across ecological and evolutionary gradients. *Proc. R. Soc. B Biol. Sci.* **289**, 20221472 (2022).
12. Gavrillets, S. Perspective: models of speciation: what have we learned in 40 years? *Evol. Int. J. Org. Evol.* **57**, 2197–2215 (2003).
13. White, N. J. *et al.* Experimental evolution of local adaptation under unidimensional and multidimensional selection. *Curr. Biol.* **32**, 1310-1318.e4 (2022).

14. Paaby, A. B. & Rockman, M. V. Cryptic genetic variation: evolution's hidden substrate. *Nat. Rev. Genet.* **15**, 247–258 (2014).
15. Zheng, J., Payne, J. L. & Wagner, A. Cryptic genetic variation accelerates evolution by opening access to diverse adaptive peaks. *Science* **365**, 347–353 (2019).
16. Bull, J. J. & Barrick, J. E. Arresting Evolution. *Trends Genet. TIG* **33**, 910–920 (2017).
17. de Kraker, M. E. A., Davey, P. G., Grundmann, H., & BURDEN study group. Mortality and hospital stay associated with resistant *Staphylococcus aureus* and *Escherichia coli* bacteremia: estimating the burden of antibiotic resistance in Europe. *PLoS Med.* **8**, e1001104 (2011).
18. Chung, H. *et al.* Rapid expansion and extinction of antibiotic resistance mutations during treatment of acute bacterial respiratory infections. *Nat. Commun.* **13**, 1231 (2022).
19. Diaz Caballero, J. *et al.* Selective Sweeps and Parallel Pathoadaptation Drive *Pseudomonas aeruginosa* Evolution in the Cystic Fibrosis Lung. *mBio* **6**, 10.1128/mbio.00981-15 (2015).
20. Scribner, M. R., Santos-Lopez, A., Marshall, C. W., Deitrick, C. & Cooper, V. S. Parallel Evolution of Tobramycin Resistance across Species and Environments. *mBio* **11**, e00932-20 (2020).
21. Baym, M., Stone, L. K. & Kishony, R. Multidrug evolutionary strategies to reverse antibiotic resistance. *Science* **351**, aad3292 (2016).
22. Andersson, D. I. & Hughes, D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat. Rev. Microbiol.* **8**, 260–271 (2010).
23. Allen, R. C., Popat, R., Diggle, S. P. & Brown, S. P. Targeting virulence: can we make evolution-proof drugs? *Nat. Rev. Microbiol.* **12**, 300–308 (2014).
24. Smith, P. A. & Romesberg, F. E. Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation. *Nat. Chem. Biol.* **3**, 549–556 (2007).
25. Andersson, D. I. The biological cost of mutational antibiotic resistance: any practical conclusions? *Curr. Opin. Microbiol.* **9**, 461–465 (2006).
26. Lázár, V. *et al.* Genome-wide analysis captures the determinants of the antibiotic cross-resistance interaction network. *Nat. Commun.* **5**, 4352 (2014).

27. Imamovic, L. & Sommer, M. O. A. Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Sci. Transl. Med.* **5**, 204ra132 (2013).
28. Jiao, Y. J., Baym, M., Veres, A. & Kishony, R. Population diversity jeopardizes the efficacy of antibiotic cycling. 082107 Preprint at <https://doi.org/10.1101/082107> (2016).
29. Nichol, D. *et al.* Antibiotic collateral sensitivity is contingent on the repeatability of evolution. *Nat. Commun.* **10**, 334 (2019).
30. Barbosa, C., Römhild, R., Rosenstiel, P. & Schulenburg, H. Evolutionary stability of collateral sensitivity to antibiotics in the model pathogen *Pseudomonas aeruginosa*. *eLife* **8**, e51481 (2019).
31. Maltas, J. & Wood, K. B. Pervasive and diverse collateral sensitivity profiles inform optimal strategies to limit antibiotic resistance. *PLOS Biol.* **17**, e3000515 (2019).
32. Barbosa, C. *et al.* Alternative Evolutionary Paths to Bacterial Antibiotic Resistance Cause Distinct Collateral Effects. *Mol. Biol. Evol.* **34**, 2229–2244 (2017).
33. Mandt, R. E. *et al.* Diverse evolutionary pathways challenge the use of collateral sensitivity as a strategy to suppress resistance. *eLife* **12**, e85023 (2023).
34. Couce, A., Rodríguez-Rojas, A. & Blázquez, J. Bypass of genetic constraints during mutator evolution to antibiotic resistance. *Proc. R. Soc. B Biol. Sci.* **282**, 20142698 (2015).
35. Oz, T. *et al.* Strength of Selection Pressure Is an Important Parameter Contributing to the Complexity of Antibiotic Resistance Evolution. *Mol. Biol. Evol.* **31**, 2387–2401 (2014).
36. Lindsey, H. A., Gallie, J., Taylor, S. & Kerr, B. Evolutionary rescue from extinction is contingent on a lower rate of environmental change. *Nature* **494**, 463–467 (2013).
37. Couce, A., Rodríguez-Rojas, A. & Blázquez, J. Determinants of Genetic Diversity of Spontaneous Drug Resistance in Bacteria. *Genetics* **203**, 1369–1380 (2016).
38. Kemble, H. *et al.* Flux, toxicity, and expression costs generate complex genetic interactions in a metabolic pathway. *Sci. Adv.* **6**, eabb2236 (2020).

39. Chou, H.-H., Delaney, N. F., Draghi, J. A. & Marx, C. J. Mapping the Fitness Landscape of Gene Expression Uncovers the Cause of Antagonism and Sign Epistasis between Adaptive Mutations. *PLOS Genet.* **10**, e1004149 (2014).
40. Lagator, M., Sarikas, S., Acar, H., Bollback, J. P. & Guet, C. C. Regulatory network structure determines patterns of intermolecular epistasis. *eLife* **6**, e28921 (2017).
41. Dellus-Gur, E. *et al.* Negative Epistasis and Evolvability in TEM-1 β -Lactamase--The Thin Line between an Enzyme's Conformational Freedom and Disorder. *J. Mol. Biol.* **427**, 2396–2409 (2015).
42. Weinreich, D. M., Delaney, N. F., Depristo, M. A. & Hartl, D. L. Darwinian evolution can follow only very few mutational paths to fitter proteins. *Science* **312**, 111–114 (2006).
43. Salverda, M. L. M. *et al.* Initial Mutations Direct Alternative Pathways of Protein Evolution. *PLOS Genet.* **7**, e1001321 (2011).
44. McInnes, R. S., McCallum, G. E., Lamberte, L. E. & van Schaik, W. Horizontal transfer of antibiotic resistance genes in the human gut microbiome. *Curr. Opin. Microbiol.* **53**, 35–43 (2020).
45. Evans, D. R. *et al.* Systematic detection of horizontal gene transfer across genera among multidrug-resistant bacteria in a single hospital. *eLife* **9**, e53886 (2020).
46. Schenk, M. F., Szendro, I. G., Salverda, M. L. M., Krug, J. & de Visser, J. A. G. M. Patterns of Epistasis between Beneficial Mutations in an Antibiotic Resistance Gene. *Mol. Biol. Evol.* **30**, 1779–1787 (2013).
47. Gonzalez, C. E. & Ostermeier, M. Pervasive Pairwise Intragenic Epistasis among Sequential Mutations in TEM-1 β -Lactamase. *J. Mol. Biol.* **431**, 1981–1992 (2019).
48. Steinberg, B. & Ostermeier, M. Shifting Fitness and Epistatic Landscapes Reflect Trade-offs along an Evolutionary Pathway. *J. Mol. Biol.* **428**, 2730–2743 (2016).
49. Knies, J. L., Cai, F. & Weinreich, D. M. Enzyme Efficiency but Not Thermostability Drives Cefotaxime Resistance Evolution in TEM-1 β -Lactamase. *Mol. Biol. Evol.* **34**, 1040–1054 (2017).

50. Salverda, M. L. M., Koomen, J., Koopmanschap, B., Zwart, M. P. & de Visser, J. A. G. M. Adaptive benefits from small mutation supplies in an antibiotic resistance enzyme. *Proc. Natl. Acad. Sci.* **114**, 12773–12778 (2017).
51. Gniadkowski, M. Evolution of extended-spectrum beta-lactamases by mutation. *Clin. Microbiol. Infect. Off. Publ. Eur. Soc. Clin. Microbiol. Infect. Dis.* **14 Suppl 1**, 11–32 (2008).
52. Ramirez, M. S., Nikolaidis, N. & Tolmasky, M. E. Rise and dissemination of aminoglycoside resistance: the aac(6')-Ib paradigm. *Front. Microbiol.* **4**, 121 (2013).
53. Di Pilato, V., Pollini, S., Miriagou, V., Rossolini, G. M. & D'Andrea, M. M. Carbapenem-resistant *Klebsiella pneumoniae*: the role of plasmids in emergence, dissemination, and evolution of a major clinical challenge. *Expert Rev. Anti Infect. Ther.* **22**, 25–43 (2024).
54. Yigit, H. *et al.* Novel carbapenem-hydrolyzing beta-lactamase, KPC-1, from a carbapenem-resistant strain of *Klebsiella pneumoniae*. *Antimicrob. Agents Chemother.* **45**, 1151–1161 (2001).
55. Queenan, A. M. & Bush, K. Carbapenemases: the Versatile β -Lactamases. *Clin. Microbiol. Rev.* **20**, 440–458 (2007).
56. Naas, T., Dortet, L. & Iorga, B. I. Structural and Functional Aspects of Class A Carbapenemases. *Curr. Drug Targets* **17**, 1006–1028 (2016).
57. Nordmann, P., Dortet, L. & Poirel, L. Carbapenem resistance in Enterobacteriaceae: here is the storm! *Trends Mol. Med.* **18**, 263–272 (2012).
58. Hobson, C. A. *et al.* *Klebsiella pneumoniae* Carbapenemase Variants Resistant to Ceftazidime-Avibactam: an Evolutionary Overview. *Antimicrob. Agents Chemother.* **66**, e0044722 (2022).
59. Naas, T. *et al.* Beta-lactamase database (BLDB) - structure and function. *J. Enzyme Inhib. Med. Chem.* **32**, 917–919 (2017).
60. Mehta, S. C., Rice, K. & Palzkill, T. Natural Variants of the KPC-2 Carbapenemase have Evolved Increased Catalytic Efficiency for Ceftazidime Hydrolysis at the Cost of Enzyme Stability. *PLOS Pathog.* **11**, e1004949 (2015).

61. Palzkill, T. Structural and Mechanistic Basis for Extended-Spectrum Drug-Resistance Mutations in Altering the Specificity of TEM, CTX-M, and KPC β -lactamases. *Front. Mol. Biosci.* **5**, (2018).
62. Gentry, L. O. Antimicrobial activity, pharmacokinetics, therapeutic indications and adverse reactions of ceftazidime. *Pharmacotherapy* **5**, 254–267 (1985).
63. Shirley, M. Ceftazidime-Avibactam: A Review in the Treatment of Serious Gram-Negative Bacterial Infections. *Drugs* **78**, 675–692 (2018).
64. Rodriguez, B. A., Giroto, J. E. & Nicolau, D. P. Ceftazidime/Avibactam and Ceftolozane/Tazobactam: Novel Therapy for Multidrug Resistant Gram Negative Infections in Children. *Curr. Pediatr. Rev.* **14**, 97–109 (2018).
65. Sharma, R., Park, T. E. & Moy, S. Ceftazidime-Avibactam: A Novel Cephalosporin/ β -Lactamase Inhibitor Combination for the Treatment of Resistant Gram-negative Organisms. *Clin. Ther.* **38**, 431–444 (2016).
66. Shields, R. K. *et al.* Emergence of Ceftazidime-Avibactam Resistance Due to Plasmid-Borne blaKPC-3 Mutations during Treatment of Carbapenem-Resistant Klebsiella pneumoniae Infections. *Antimicrob. Agents Chemother.* **61**, e02097-16 (2017).
67. Sun, Z. *et al.* Klebsiella pneumoniae carbapenemase variant 44 acquires ceftazidime-avibactam resistance by altering the conformation of active-site loops. *J. Biol. Chem.* **300**, 105493 (2024).
68. Cano, A. V., Rozhoňová, H., Stoltzfus, A., McCandlish, D. M. & Payne, J. L. Mutation bias shapes the spectrum of adaptive substitutions. *Proc. Natl. Acad. Sci.* **119**, e2119720119 (2022).
69. Woodford, N. *et al.* 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 (2004).
70. Kazmierczak, K. M. *et al.* Global Dissemination of blaKPC into Bacterial Species beyond Klebsiella pneumoniae and In Vitro Susceptibility to Ceftazidime-Avibactam and Aztreonam-Avibactam. *Antimicrob. Agents Chemother.* **60**, 4490–4500 (2016).

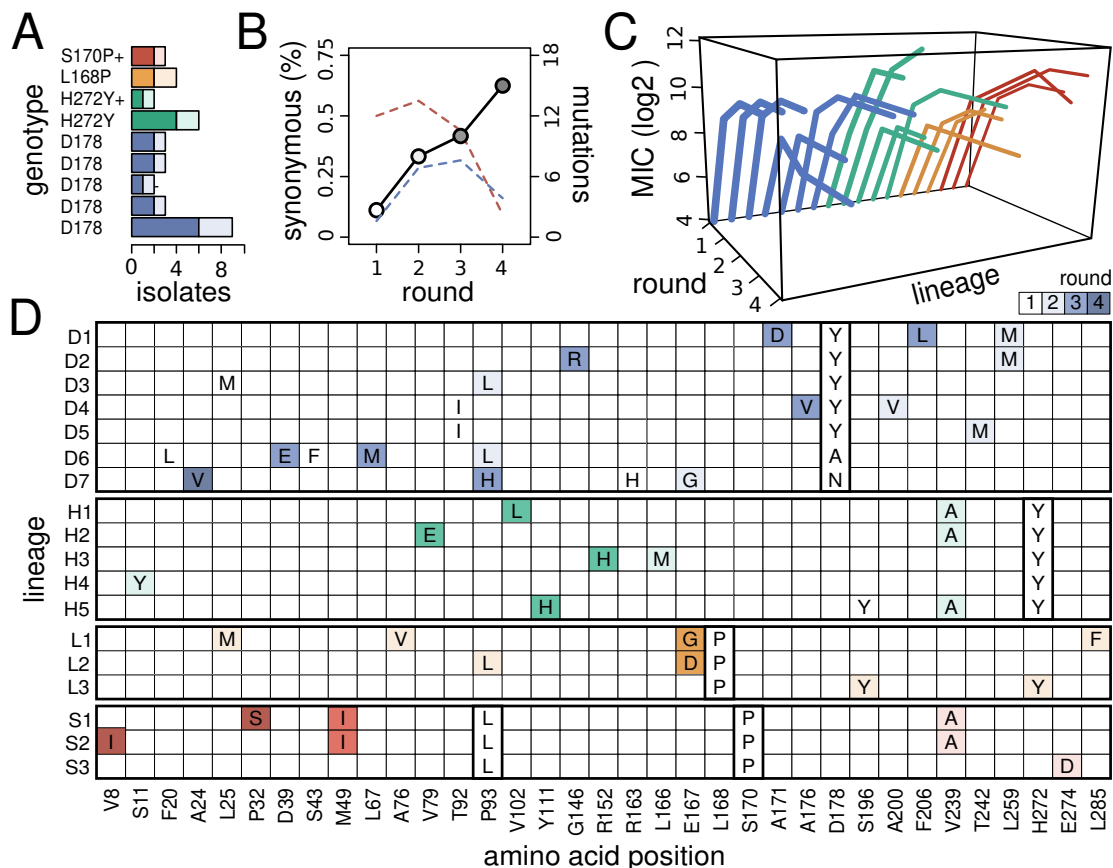
71. Szili, P. *et al.* Rapid Evolution of Reduced Susceptibility against a Balanced Dual-Targeting Antibiotic through Stepping-Stone Mutations. *Antimicrob. Agents Chemother.* **63**, e00207-19 (2019).
72. Rosenkilde, C. E. H. *et al.* Collateral sensitivity constrains resistance evolution of the CTX-M-15 β -lactamase. *Nat. Commun.* **10**, 618 (2019).
73. Goulart, C. P. *et al.* Designing antibiotic cycling strategies by determining and understanding local adaptive landscapes. *PLoS One* **8**, e56040 (2013).
74. Mira, P. M. *et al.* Rational Design of Antibiotic Treatment Plans: A Treatment Strategy for Managing Evolution and Reversing Resistance. *PLoS ONE* **10**, e0122283 (2015).
75. Turner, J. *et al.* The Chemical Relationship Among Beta-Lactam Antibiotics and Potential Impacts on Reactivity and Decomposition. *Front. Microbiol.* **13**, (2022).
76. Kinsler, G., Li, Y., Sherlock, G. & Petrov, D. A. A shift from pleiotropic to modular adaptation revealed by a high-resolution two-step adaptive walk. 2024.04.17.589938 Preprint at <https://doi.org/10.1101/2024.04.17.589938> (2024).
77. Cooper, T. F., Ostrowski, E. A. & Travisano, M. A negative relationship between mutation pleiotropy and fitness effect in yeast. *Evol. Int. J. Org. Evol.* **61**, 1495–1499 (2007).
78. Nikaido, H. & Normark, S. Sensitivity of *Escherichia coli* to various beta-lactams is determined by the interplay of outer membrane permeability and degradation by periplasmic beta-lactamases: a quantitative predictive treatment. *Mol. Microbiol.* **1**, 29–36 (1987).
79. López, C., Delmonti, J., Bonomo, R. A. & Vila, A. J. Deciphering the evolution of metallo- β -lactamases: A journey from the test tube to the bacterial periplasm. *J. Biol. Chem.* **298**, 101665 (2022).
80. Guin, D. & Gruebele, M. Weak Chemical Interactions That Drive Protein Evolution: Crowding, Sticking, and Quinary Structure in Folding and Function. *Chem. Rev.* **119**, 10691–10717 (2019).

81. Meini, M.-R., Tomatis, P. E., Weinreich, D. M. & Vila, A. J. Quantitative Description of a Protein Fitness Landscape Based on Molecular Features. *Mol. Biol. Evol.* **32**, 1774–1787 (2015).
82. López, C., Ayala, J. A., Bonomo, R. A., González, L. J. & Vila, A. J. Protein determinants of dissemination and host specificity of metallo- β -lactamases. *Nat. Commun.* **10**, 3617 (2019).
83. Socha, R. D., Chen, J. & Tokuriki, N. The Molecular Mechanisms Underlying Hidden Phenotypic Variation among Metallo- β -Lactamases. *J. Mol. Biol.* **431**, 1172–1185 (2019).
84. Mehlhoff, J. D. *et al.* Collateral fitness effects of mutations. *Proc. Natl. Acad. Sci.* **117**, 11597–11607 (2020).
85. Mehlhoff, J. D. & Ostermeier, M. Genes Vary Greatly in Their Propensity for Collateral Fitness Effects of Mutations. *Mol. Biol. Evol.* **40**, msad038 (2023).
86. González, L. J., Bahr, G., González, M. M., Bonomo, R. A. & Vila, A. J. In-cell kinetic stability is an essential trait in metallo- β -lactamase evolution. *Nat. Chem. Biol.* **19**, 1116–1126 (2023).
87. Fröhlich, C. *et al.* Cryptic β -Lactamase Evolution Is Driven by Low β -Lactam Concentrations. *mSphere* **6**, e00108-21 (2021).
88. Tokuriki, N. *et al.* Diminishing returns and tradeoffs constrain the laboratory optimization of an enzyme. *Nat. Commun.* **3**, 1257 (2012).
89. Morley, K. L. & Kazlauskas, R. J. Improving enzyme properties: when are closer mutations better? *Trends Biotechnol.* **23**, 231–237 (2005).
90. Barnes, M. D. *et al.* Klebsiella pneumoniae Carbapenemase-2 (KPC-2), Substitutions at Ambler Position Asp179, and Resistance to Ceftazidime-Avibactam: Unique Antibiotic-Resistant Phenotypes Emerge from β -Lactamase Protein Engineering. *mBio* **8**, e00528-17 (2017).
91. Alsenani, T. A. *et al.* Structural Characterization of the D178N and D178Y Variants of KPC-2 β -Lactamase: Ω -Loop Destabilization as a Mechanism of Resistance to Ceftazidime-Avibactam. *Antimicrob. Agents Chemother.* **66**, e02414-21.

92. Tooke, C. L. *et al.* Natural variants modify *Klebsiella pneumoniae* carbapenemase (KPC) acyl-enzyme conformational dynamics to extend antibiotic resistance. *J. Biol. Chem.* **296**, 100126 (2021).
93. Pál, C., Papp, B. & Lázár, V. Collateral sensitivity of antibiotic-resistant microbes. *Trends Microbiol.* **23**, 401–407 (2015).
94. Gullberg, E. *et al.* Selection of resistant bacteria at very low antibiotic concentrations. *PLoS Pathog.* **7**, e1002158 (2011).
95. Ribeiro, A. R., Sures, B. & Schmidt, T. C. Cephalosporin antibiotics in the aquatic environment: A critical review of occurrence, fate, ecotoxicity and removal technologies. *Environ. Pollut. Barking Essex 1987* **241**, 1153–1166 (2018).
96. Baquero, F. & Negri, M. C. Selective compartments for resistant microorganisms in antibiotic gradients. *BioEssays News Rev. Mol. Cell. Dev. Biol.* **19**, 731–736 (1997).
97. Baier, F. *et al.* Cryptic genetic variation shapes the adaptive evolutionary potential of enzymes. *eLife* **8**, e40789 (2019).
98. Bloom, J. D. *et al.* Evolution favors protein mutational robustness in sufficiently large populations. *BMC Biol.* **5**, 29 (2007).
99. Besenmatter, W., Kast, P. & Hilvert, D. Relative tolerance of mesostable and thermostable protein homologs to extensive mutation. *Proteins* **66**, 500–506 (2007).
100. Couce, A. *et al.* Changing fitness effects of mutations through long-term bacterial evolution. *Science* **383**, eadd1417 (2024).
101. Leiby, N. & Marx, C. J. Metabolic Erosion Primarily Through Mutation Accumulation, and Not Tradeoffs, Drives Limited Evolution of Substrate Specificity in *Escherichia coli*. *PLOS Biol.* **12**, e1001789 (2014).
102. Card, K. J., LaBar, T., Gomez, J. B. & Lenski, R. E. Historical contingency in the evolution of antibiotic resistance after decades of relaxed selection. *PLoS Biol.* **17**, e3000397 (2019).
103. Tenaillon, O. *et al.* The molecular diversity of adaptive convergence. *Science* **335**, 457–461 (2012).

104. Rodríguez-Verdugo, A., Gaut, B. S. & Tenailon, O. Evolution of *Escherichia coli* rifampicin resistance in an antibiotic-free environment during thermal stress. *BMC Evol. Biol.* **13**, 50 (2013).
105. Pérez, M. *et al.* Mutual regulation between SIAH2 and DYRK2 controls hypoxic and genotoxic signaling pathways. *J. Mol. Cell Biol.* **4**, 316–330 (2012).

769 FIGURES



770

771 **Figure 1.- Mutational pathways recovered during KPC-2 directed evolution for**

772 **increased CAZ resistance.** (A) Abundance of the nine unique genotypes retrieved

773 among the initial 22 colonies isolated in Round 1. Colors indicate grouping into four

774 categories, according to mutations in positions previously associated with CAZ

775 resistance. The presumed driver mutation is labeled on the y-axis, with a '+' sign

776 indicating additional accessory mutations (see Table S1). Darker shades represent total

777 abundance across replicates, while lighter shades indicate independent occurrences. (B)

778 Proportion of synonymous mutations across rounds (solid black line with circles; darker

779 gray shades represent later rounds). The secondary y-axis displays the total number of

780 synonymous (blue dashed line) and non-synonymous mutations (red dashed line). (C)

781 Diversity of CAZ resistance trajectories across rounds and lineages, grouped and

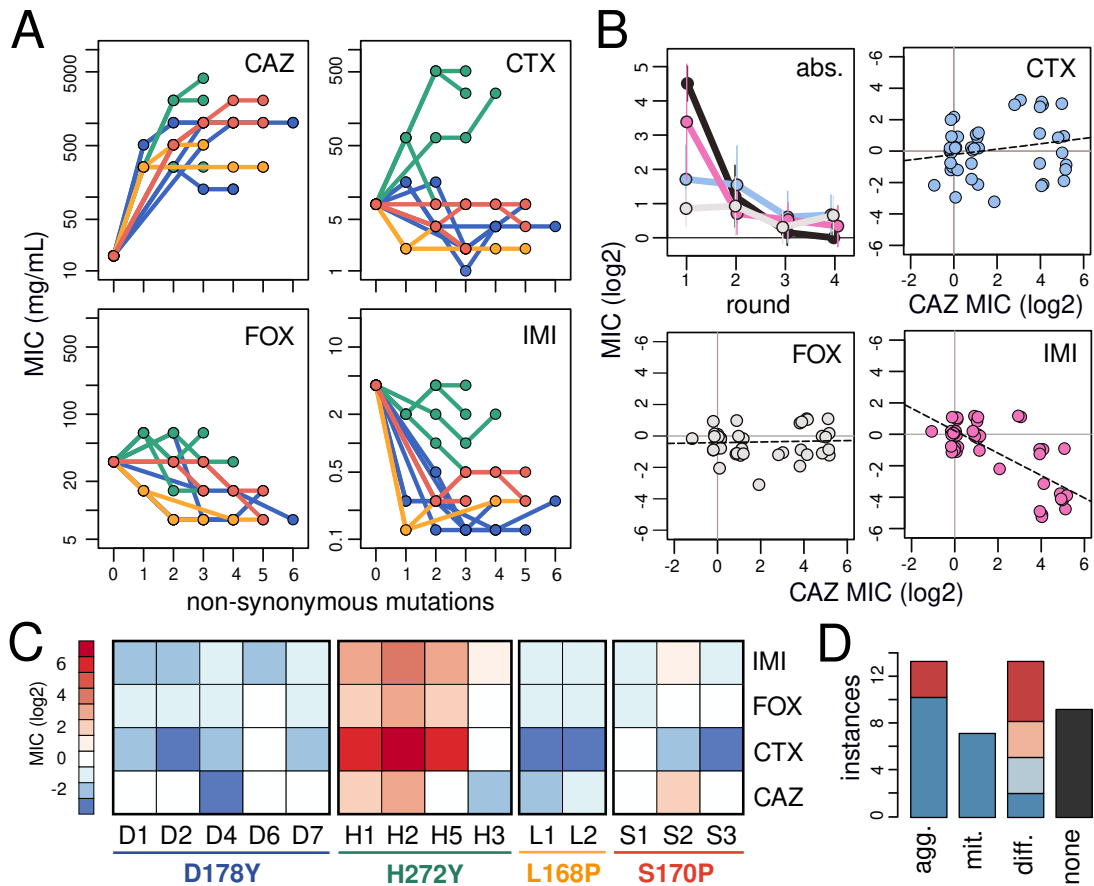
782 colored as in (A). (D) Horizontal alignment of the 18 lineages studied, highlighting only

783 positions with mutations. Lineages are arranged, grouped, and colored as in (A), with

784 darker shades representing later rounds. Presumed driver mutations are highlighted with

785 thick-lined squares to underscore they are the basis for defining each group.

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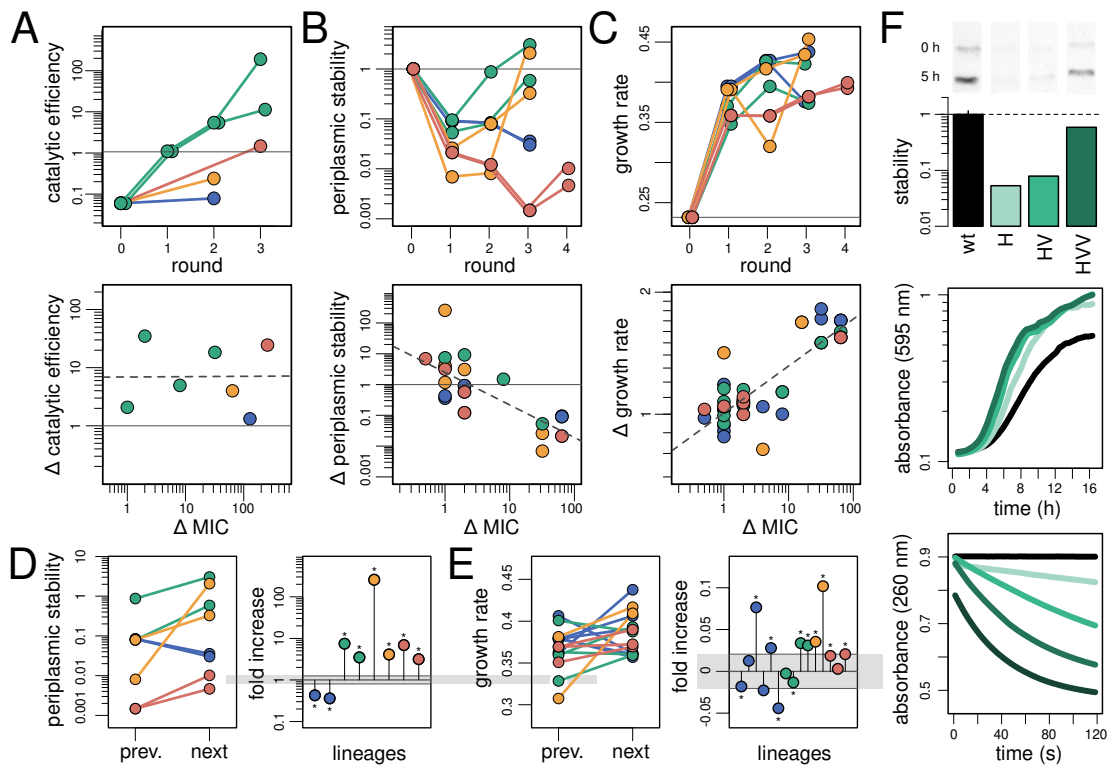


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788 **Figure 2.-** Collateral sensitivity toward other β -lactams rapidly diverges during CAZ-
 789 resistance evolution. (A) Resistance trajectories against the number of non-synonymous
 790 mutations accumulated across rounds for each lineage, with colors indicating the
 791 grouping based on positions associated with CAZ resistance: blue (D178Y), green
 792 (H272Y), yellow (L168P), and red (S170P). Panels show MIC measurements for CAZ,
 793 cefotaxime (CTX), ceftioxin (FOX), and imipenem (IMI), as labeled in the upper right
 794 corner. (B) Patterns of resistance across rounds. The first panel shows the average
 795 overall change in magnitude for each β -lactam, with error bars representing standard
 796 deviation. Colors denote averages for CAZ (black), CTX (cyan), FOX (grey) and IMI
 797 (pink). The remaining panels show correlations between individual MIC changes for
 798 CAZ and the other antibiotics, with dashed lines indicating the best fit from linear
 799 regression. (C) Variation in collateral sensitivity among and within lineages. Lineage
 800 labels are color-coded as in (A). Resistance values for endpoint isolates are represented
 801 following a color gradient (blue, increased sensitivity; red, cross-resistance). Note
 802 values denote relative variation compared to all lineages. (D) Divergence in collateral
 803 sensitivity along mutational pathways. Values indicate the number of lineages for which
 804 the full path has aggravating, mitigating, different, or no effects on the first-step mutant

805 resistance. Blue indicates that the first step caused increased sensitivity; red indicates it
806 caused resistance. Shaded colors represent cases where the endpoint profile is neutral
807 compared to the wild-type.

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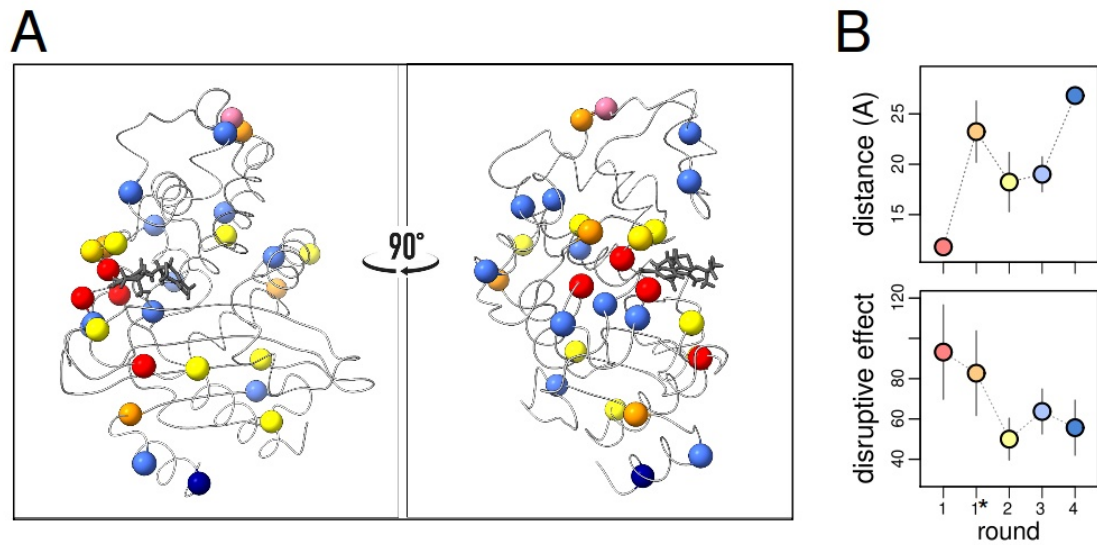


809

810 **Figure 3.-** Molecular traits rapidly diverge during CAZ-resistance evolution. (A) Top
 811 panel: catalytic efficiency (k_{cat}/K_m) across rounds for selected enzyme variants,
 812 expressed as fold change relative to the ancestral KPC-2 enzyme. Colors indicate initial
 813 CAZ resistance mutations: blue (D178Y), green (H272Y), yellow (L168P), and red
 814 (S170P). Bottom panel: correlation between individual changes in k_{cat}/K_m and MIC, with
 815 best-fit (dashed line) and ancestral value (solid line). (B) Top panel: stability of enzyme
 816 abundance in the periplasm across rounds for the same variants as in Figure 2. Values
 817 represent Western blot intensity changes over five hours of growth, relative to ancestral
 818 KPC-2, expressed relative to the ancestral KPC-2 enzyme (see F). Color coding as
 819 above. Bottom panel: correlation between individual changes in periplasmic stability
 820 and MIC. Dashed line indicates the best fit from linear regression; horizontal solid line
 821 stability of the ancestral enzyme. (C) Top panel: growth rates in sub-MIC concentrations
 822 of CAZ across rounds for the same variants as in (B). Bottom panel: correlation
 823 between individual changes in growth rate and MIC. Dashed and solid lines show best
 824 fit and reference values as above. (D) Change in periplasmic stability between
 825 consecutive mutants within plateaus (left). Change expressed as fold increase (right);
 826 gray bar represents measurement uncertainty (mean \pm 2 x standard deviation for the
 827 wild-type). Asterisks indicate statistical significance. (E) Similar analysis as in (D), but

828 showing changes in growth rate between consecutive mutants within each plateau. (F)
829 Top panel: example Western blot quantifying periplasmic stability, with a bar plot
830 showing the log intensity ratio of initial (1 h) and final (5 h) bands for each variant.
831 Middle panel: example growth rates in sub-MIC CAZ concentrations for the same
832 variants. Bottom panel: example CAZ concentration decay from the colorimetric assay
833 for the same variants.

834



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836 **Figure 4.- Position and properties of accumulated mutations across rounds. (A)**

837 Diagram of the KPC-2 β -lactamase backbone with CAZ bound at the active site (shown
838 as sticks). Positions where mutations were observed are represented as spheres, colored

839 according to the round in which they arose: red (Round 1, presumably driver

840 mutations), orange (Round 1, accessory), yellow (Round 2), light blue (Round 3), dark

841 blue (Round 4). The purple sphere marks the P94 substitution, present in all rounds of

842 mutagenesis. **(B)** Average atomic distance of mutations from CAZ's center of mass

843 (top), and their Grantham score (physiochemical distance) as a proxy of their disruptive

844 effect per round. Circle colors follow the same scheme as in (A).

845

line	Round 1			Round 2			Round 3			Round 4		
	nucleotide	amino acid	MIC (µg/ml)	nucleotide	amino acid	MIC (µg/ml)	nucleotide	amino acid	MIC (µg/ml)	nucleotide	amino acid	MIC (µg/ml)
D1	g532t	D178Y	512	<u><i>c735t</i></u> , <i>t775a</i>	L259M	1024	<i>c512a</i> , <i>t618a</i> , <u><i>c735t</i></u>	A171D, F206L	1024			
D2	g532t	D178Y	512	<u><i>c735t</i></u> , <i>t775a</i>	L259M	1024	g436a	G146R	1024			
D3	<i>c73a</i> , <u><i>a105g</i></u> , <u><i>c204t</i></u> , g532t	L25M, D178Y	512	c278t, <u><i>g666a</i></u>	P93L, L25M, D178Y	1024	<u><i>g546t</i></u>	none	1024			
D4	c275t, g532t	T92I, D178Y	256	c600t, <u><i>c654t</i></u>	A200V	128	<u><i>c361t</i></u> , c527t	A176V	128	<u><i>g267a</i></u>	none	128
D5	c275t, g532t	T92I, D178Y	256	c725t	T92I, D178Y, T242M	256	<u><i>t771a</i></u>	none	256			
D6	<i>t58c</i> , c128t, a533c	F20L, S43F, D178A	512	<u><i>c278t</i></u> , c279t, <u><i>g300a</i></u>	P93L	1024	c117g, c199a, <u><i>t775c</i></u>	D39E, L67M	1024	<u><i>c507t</i></u>	none	1024
D7	g488a, g532a	R163H, D178N	256	a500g	E167G	1024	c278a	P93H	1024	c71t	A24V	1024
H1	c814t	H272Y	256	<u><i>t716c</i></u>	V239A	2048	g304c	V102L	2048			
H2	c814t	H272Y	256	<u><i>t716c</i></u>	V239A	2048	<i>t236a</i>	V79E	4096	wt	wt	4096
H3	c814t	H272Y	256	<u><i>c496a</i></u> , <u><i>t643c</i></u>	L166M	256	<u><i>g455a</i></u>	R152H	256			
H4	c814t	H272Y	256	c32a	S11Y, H272Y	256						
H5	c587a, c814t	S196Y, H272Y	512	<u><i>t716c</i></u>	V239A	1024	<i>t331c</i> , <u><i>t621c</i></u>	Y111H	1024	<u><i>c195t</i></u>	none	1024
L1	i503c	L168P	256	<i>c73a</i> , c227t, <u><i>c663t</i></u> , c853t	L25M, A76V, L285F	256	a500g, <u><i>g666a</i></u>	E167G	256	<u><i>t738c</i></u>	none	256
L2	i503c	L168P	256	c278t	P93L	512	g501t	E167D	512			
L3	i503c	L168P	256	c587a, c814t	S196Y, H272Y	512	wt	wt	512			
S1	c278t, i508c	P93L, S170P	512	<u><i>a42g</i></u> , <u><i>t716c</i></u>	V239A	1024	g147a, <u><i>t768a</i></u>	M49I	2048	c94t	P32S	1024
S2	c278t, i508c	P93L, S170P	512	<u><i>a42g</i></u> , <u><i>t716c</i></u>	V239A	1024	g147a, <u><i>t768a</i></u>	M49I	2048	<u><i>g22a</i></u> , <u><i>c496t</i></u>	V8I	2048
S3	c278t, i508c	P93L, S170P	512	<u><i>g822t</i></u> , <u><i>a881g</i></u>	E274D	1024	<u><i>g675c</i></u> , <u><i>g879a</i></u>	none	1024			

846

847 **Table S1. Mutations recovered across different rounds of directed evolution.**

848 Lineages are grouped by the presumed first beneficial mutation, color-coded as blue
849 (D178Y), green (H272Y), yellow (L168P), and red (S170P). Each round includes three
850 columns showing changes at the nucleotide level in the *blaKPC* gene, at the amino acid
851 level in the KPC protein, and MIC values for CAZ. Synonymous mutations are
852 indicated by underlined, italicized nucleotide changes.

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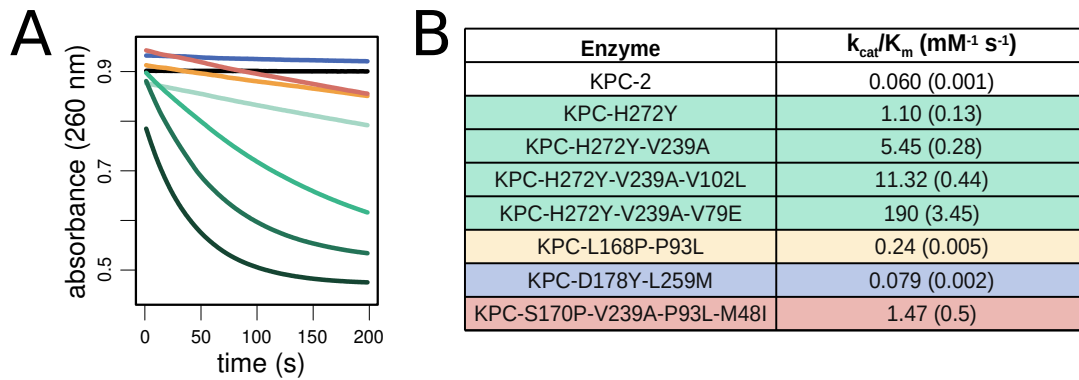
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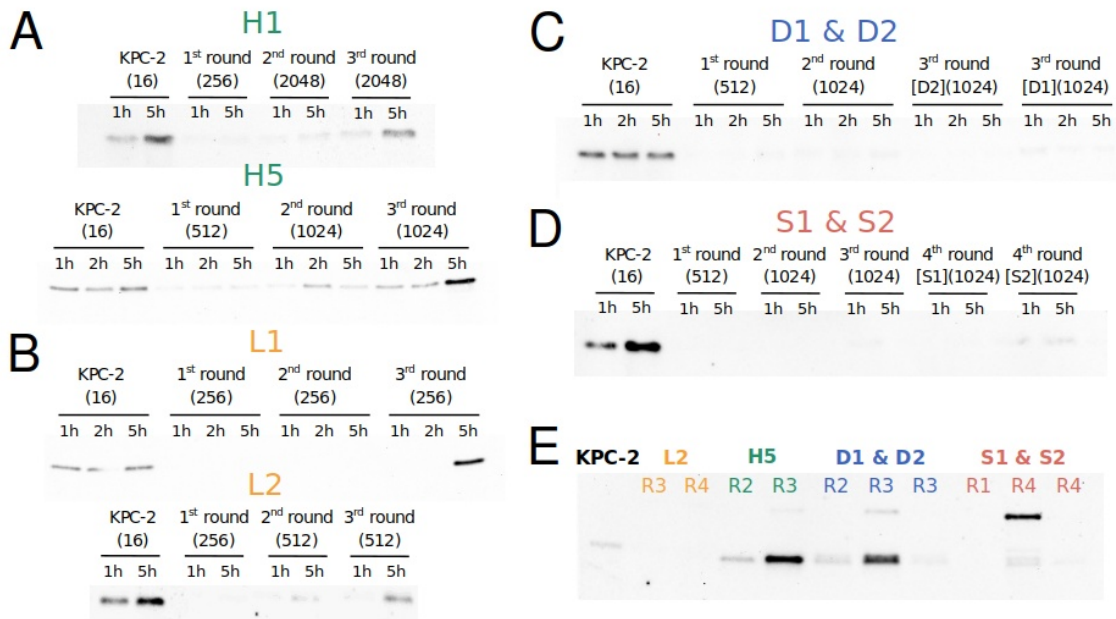
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868 **Figure S1. Progress curves of CAZ hydrolysis from a colorimetric assay.** All
869 reactions were performed with 500 nM enzyme and 50 μM ceftazidime. Hydrolysis of
870 ceftazidime results in a decrease in absorbance at 260 nm. Curves correspond to: KPC-2
871 (black), KPC-D178P-L260M (blue), KPC-L168P-P94L (yellow), KPC-S170P-V240A-
872 P94L-M49I (orange), KPC-H272Y (very light green), KPC-H272Y-V240A (light
873 green), KPC-H272Y-V240A-V103L (green), and KPC-H272Y-V240A-V80E (dark
874 green). **(B)** Steady-state kinetic parameters for CAZ hydrolysis for ancestral KPC-2 and
875 its variants.

876



877

878 **Figure S2. Western blot of KPC-2 and its variants in periplasmic extracts of *E. coli***
 879 **TOP10.** (A-D) Periplasmic abundance of representative lineages from groups H272
 880 (green), L168 (yellow), D178 (blue), and S170 (red) after 1 and 5 hours of growth. (E)
 881 Immunoblot of selected KPC variants in the periplasm of *E. coli* TOP10 after 10 hours
 882 of incubation. Colors as above.

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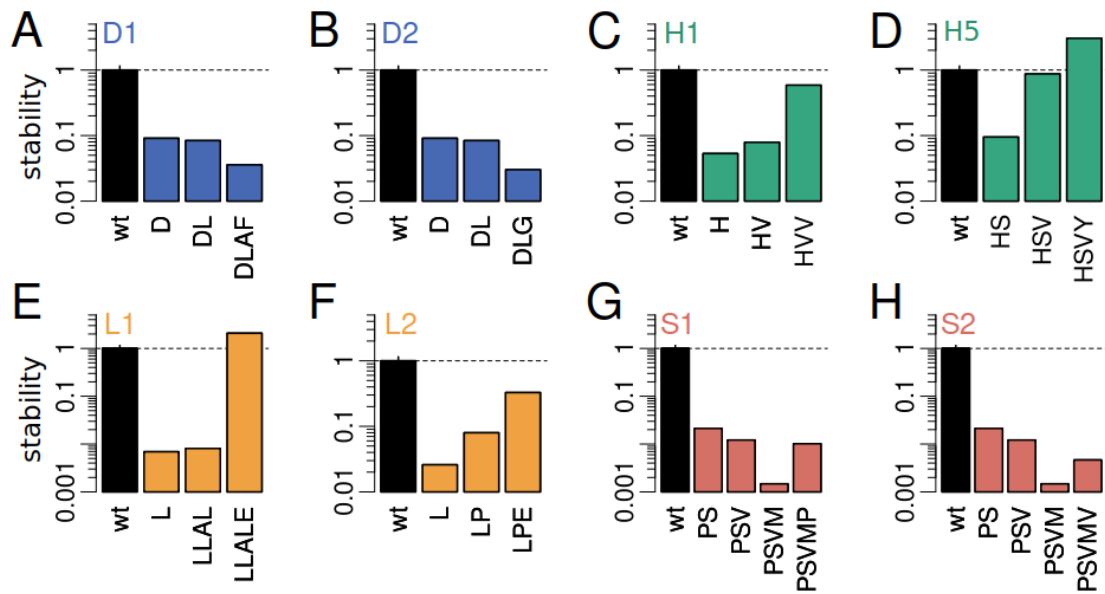
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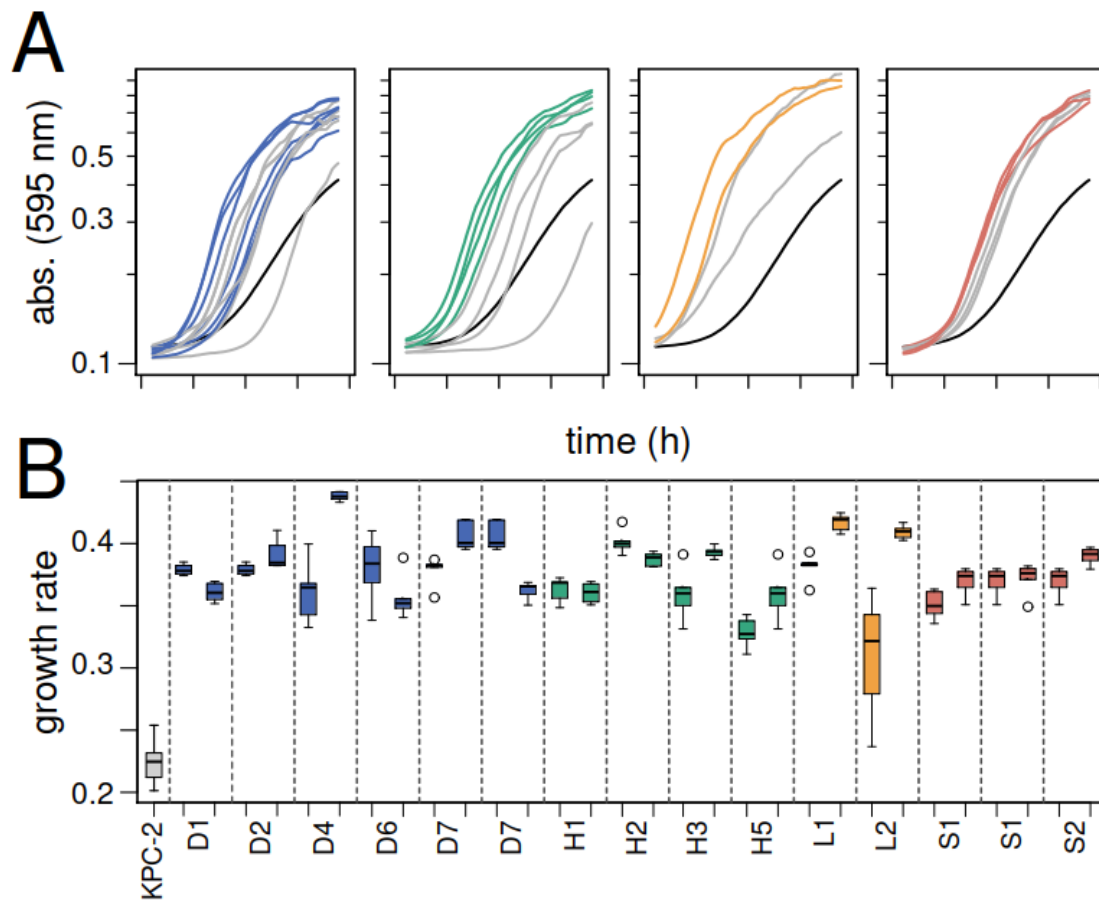
901 **Figure S3.- Periplasmic stability of selected variants.** Bar plots show the log intensity

902 ratio of initial (1 h) and final (5 h) bands for each variant, quantified from the Western

903 blots in Figure S2. Lineage labels are in the upper left. Colors as in Figure S2.

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907 **Figure S4. Growth rates of consecutive mutants within plateaus. (A)** Optical density
908 changes over 5 hours of growth. The black line represents the strain with wild-type
909 KPC-2, gray lines represent earlier plateau strains, and colored lines represent
910 subsequent strains. **(B)** Boxplot summarizing maximum growth rates from the growth
911 curves above.