View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Open Research Exeter

1 **Title:** Alternative evolutionary paths to bacterial antibiotic resistance cause distinct
2 collateral effects
3
4
5 **Authors:** C. Barbosa[1], V. Trebosc[2], C. Kemmer[2], P. Rosenstiel[3], R. Beardmore[4], H.
6 Schulenburg[1*] and G. Jansen[1*]
7
8 **Affiliations:**
9 [1]Evolutionary Ecology and Genetics, Zoological Institute, CAU Kiel, Kiel 24118,
10 Germany.
11 [2]BioVersys AG, Basel CH-4057, Switzerland.
12 [3]Institute of Clinical Molecular Biology (IKMB), CAU Kiel, Kiel 24105, Germany.
13 [4]Biosciences, Geoffrey Pope Building, University of Exeter, Exeter EX4 4QD, United
14 Kingdom.
15
16 *Correspondence to hschulenburg@zoologie.uni-kiel.de or gjansen@zoologie.uni-
17 kiel.de
18
19

20 **Abstract (max. 250 words)**
21 When bacteria evolve resistance against a particular antibiotic, they may
22 simultaneously gain increased sensitivity against a second one. Such collateral
23 sensitivity may be exploited to develop novel, sustainable antibiotic treatment strategies
24 aimed at containing the current, dramatic spread of drug resistance. To date, the
25 presence and molecular basis of collateral sensitivity has only been studied in few
26 bacterial species and is unknown for opportunistic human pathogens such as
27 *Pseudomonas aeruginosa*. In the present study, we assessed patterns of collateral
28 effects by experimentally evolving 160 independent populations of *P. aeruginosa* to
29 high levels of resistance against eight commonly used antibiotics. The bacteria evolved
30 resistance rapidly and expressed both collateral sensitivity and cross-resistance. The
31 pattern of such collateral effects differed to those previously reported for other bacterial
32 species, suggesting inter-specific differences in the underlying evolutionary trade-offs.
33 Intriguingly, we also identified contrasting patterns of collateral sensitivity and cross-
34 resistance among the replicate populations adapted to the same drug. Whole-genome
35 sequencing of 81 independently evolved populations revealed distinct evolutionary
36 paths of resistance to the selective drug, which determined whether bacteria became
37 cross-resistant or collaterally sensitive towards others. Based on genomic and
38 functional genetic analysis, we demonstrate that collateral sensitivity can result from
39 resistance mutations in regulatory genes such as *nalC* or *mexZ*, which mediate
40 aminoglycoside sensitivity in β-lactam-adapted populations, or the two-component
41 regulatory system gene *pmrB*, which enhances penicillin sensitivity in gentamicin-
42 resistant populations. Our findings highlight substantial variation in the evolved
43 collateral effects among replicates, which in turn determine their potential in antibiotic
44 therapy.
45

46 **Introduction**

47 Bacteria have the potential to rapidly adapt to virtually any natural or laboratory
48 environment (Kussell 2013). The long-term evolution experiment with *Escherichia coli*
49 (LTEE) has shown that even in simple constant environments, bacteria can achieve
50 comprehensive fitness increases of about 25% within the first 2,000 generations (Lenski
51 et al. 1991). Although the LTEE populations show reduced adaptation rates at later time
52 points (e.g., after 50,000 generations), they still continue to accumulate an almost
53 constant number of new beneficial mutations (Barrick et al. 2009; Tenaillon et al.
54 2016). Thus, bacteria can adapt rapidly to new challenges and subsequently continue to
55 optimize their fitness. Such remarkable adaptive potential was also observed under
56 more challenging conditions: Using evolution experiments with antibiotics, *E. coli*
57 evolved high levels of drug resistance through the step-wise accumulation of multiple
58 mutations when drug concentrations increased over time (Toprak et al. 2012) or across
59 space (Baym, Lieberman, et al. 2016). Bacteria also readily adapted when they were
60 challenged with two antibiotics simultaneously (Chait et al. 2007; Hegreness et al.
61 2008; Michel et al. 2008; Pena-Miller et al. 2013), or sequentially (Kim et al. 2014;
62 Fuentes-Hernandez et al. 2015; Roemhild et al. 2015).

63

64 Rapid bacterial adaptation to new environments often involves evolutionary trade-offs
65 in the form of reduced fitness under alternative growth conditions (Kussell 2013). In
66 the case of antibiotic resistance evolution, two types of trade-offs (or costs) are
67 commonly observed: (i) evolved resistance is costly in the absence of the drugs, thus
68 generating growth deficiencies relative to the susceptible ancestor (Andersson and
69 Hughes 2010; Melnyk et al. 2015), and (ii) resistance mutations may exacerbate
70 susceptibility against others (i.e. collateral sensitivity (Szybalski and Bryson 1952; Pál
71 et al. 2015); also referred to as hypersensitivity, or negative cross-resistance in previous
72 publications). However, adaptive mutations do not always entail a cost but instead may
73 increase resistance against other antibiotics (i.e., collateral resistance or cross-
74 resistance); thus favoring multi-drug resistance.

75

76 The phenomenon of collateral sensitivity was first described in the 1950s in a study by
77 Szybalski and Bryson, in which the authors tested if experimentally evolved resistant
78 *E. coli* was less, equally or more sensitive to previously unmet drugs (Szybalski and
79 Bryson 1952). Despite finding that cross-resistance was much more prevalent than

80 collateral sensitivity, the authors hypothesized that these rare cases could then be
81 exploited by rationally using more than one drug during treatment of resistant clinical
82 strains. The employment of drug pairs that produce reciprocal collateral sensitivity
83 might trap bacteria in an evolutionary 'double-bind', thus improving treatment efficacy
84 and decreasing the evolution of resistance. This idea was more recently tested by
85 exposing bacteria to such drug pairs being deployed sequentially (Imamovic and
86 Sommer 2013; Kim et al. 2014; Fuentes-Hernandez et al. 2015; Roemhild et al. 2015)
87 or simultaneously (Munck et al. 2014; Evgrafov et al. 2015). Additionally, several other
88 studies have further evaluated what factors could help to predict the changes in drug
89 sensitivity in experimentally evolved resistant *E. coli.* These showed that the strength
90 of selection and the chemogenomic profile similarity between antibiotics play
91 significant roles in the evolution of resistance and hence influence the patterns of cross-
92 resistance and hypersensitivity (Lázár et al. 2013; Lázár et al. 2014; Oz et al. 2014).
93
94 To fully determine the importance of such trade-offs during bacterial adaptation and
95 also their therapeutic potential, the patterns of collateral resistance/sensitivity observed
96 in *E. coli* need to be assessed in other, clinically relevant bacterial taxa, including those
97 known to possess high adaptive capacity such as members of the genus *Pseudomonas*.
98 This group of bacteria are able to colonize and thrive in a plethora of niches (Nikel et
99 al. 2014), and act as prominent plant and human pathogens (Loper et al. 2012;
100 Rodríguez-Rojas et al. 2012; Balasubramanian et al. 2013). For instance, the
101 opportunistic human pathogen *P. aeruginosa* is commonly associated with hospital-
102 acquired infections, and it is a major cause of chronic lung disease, including the
103 ultimately fatal infections in cystic fibrosis patients (Govan and Deretic 1996; Arruda
104 et al. 1999; Kang et al. 2003; Folkesson et al. 2012). Its success as an opportunistic
105 pathogen can be largely attributed to its vast array of virulence factors, including the
106 production of alginate to form biofilms, its ability to survive oxidative stress, and the
107 availability of various secretion systems (Hauser 2009; Ma et al. 2009; Burrows 2012;
108 Jimenez et al. 2012; Korotkov et al. 2012). Moreover, it carries a large array of intrinsic
109 antibiotic resistance mechanisms and an exceptional potential to acquire resistance,
110 both *de novo* or horizontally transferred (Arruda et al. 1999; Carmeli et al. 1999;
111 Hancock and Speert 2000; Poole 2001; Drenkard and Ausubel 2002; Livermore 2002;
112 Overhage et al. 2008; Breidenstein et al. 2011). To date there is neither information on
113 the evolution of drug sensitivity trade-offs in *P. aeruginosa,* nor on its genomic

114 underpinnings. To rectify this knowledge gap, we experimentally selected 160 highly
115 resistant populations of *P. aeruginosa* PA14 (plus 20 control populations) and
116 evaluated the possibility of evolutionary trade-offs in the form of fitness deficiencies in
117 the absence of antibiotics and hypersensitivity to other drugs. We assessed the
118 underlying molecular mechanisms of such trade-offs through whole-genome
119 sequencing of 81 evolved resistant populations.
120
121 **Results and discussion**
122 **Rapid evolution of antibiotic resistance**
123 We used experimental evolution to generate independent populations of *P. aeruginosa*
124 PA14 with significantly increased resistance against eight different antibiotics. We
125 challenged 20 isogenic populations against increasing concentrations of one out of eight
126 different drugs (for abbreviations of all antibiotics see table 1), and included an
127 evolutionary control grown in media only, resulting in a total of 180 independent
128 populations. Experiments were initiated at half the concentration inhibiting >90% of
129 growth (abbreviated IC90), as determined for the ancestral PA14. Populations were
130 then serially transferred every 12 h for 24 days (48 transfers; approximately 58
131 generations) until reaching around 40 times the IC90, or until less than half of the
132 starting populations from a given antibiotic were still growing (fig. 1A). In the cases of
133 DOR, IMI, and CAR, more than half of the populations went extinct at transfers 20, 28,
134 and 40, respectively; we accordingly isolated 10 populations adapted to the 2x, 5x and
135 17x environments for further analysis. In all other cases, we randomly chose 10
136 surviving populations per antibiotic for subsequent characterization. Frozen
137 populations adapted to GEN could not be recovered after thawing; we could only revive
138 10 of those adapted to the 5x environment. Altogether we obtained 80 populations
139 adapted to 8 different antibiotics and 10 additional ones adapted to media only.
140
141 **Table 1.** List of antibiotics used during selection experiments.

Functional target	Class	Drug	Abbreviation
DNA repair	Quinolones	Ciprofloxacin	CIP
Protein synthesis	Aminoglycosides	Gentamicin	GEN
		Streptomycin	STR
Cell wall synthesis	Penicillins	Piperacillin + Tazobactam	PIT
		Carbenicillin	CAR

Carbapenems	Doripenem	DOR
	Imipenem	IMI
Cephalosporins	Cefsulodin	CEF

142

143 For the selected populations, we quantified the changes in resistance to the respective
144 drug used during the evolution experiment (see Materials and Methods). *P. aeruginosa*
145 could rapidly (within 58 generations or less) reach resistance levels of at least 32 times
146 the IC90 of the ancestral PA14 for most antibiotics (fig. 1B-I). Interestingly, we
147 observed substantial variation within particular drug treatments, suggesting different
148 routes of adaptation to the antibiotic. Similar increases in drug resistance were found in
149 a recent study with the related *P. aeruginosa* strain PAO1 (Cabot et al. 2016),
150 highlighting the adaptive potential of this species. The same study also revealed in most
151 cases an association of increased resistance with genomic changes in well-known
152 resistance pathways. *P. aeruginosa*'s resistance thus appears to be achieved fast
153 through diverse mechanisms. Several comparable *E. coli* evolution experiments
154 resulted in similar levels of resistance, although variation between populations adapted
155 to the same drug was less pronounced than observed here for *P. aeruginosa* (Imamovic
156 and Sommer 2013; Lázár et al. 2013; Oz et al. 2014).
157

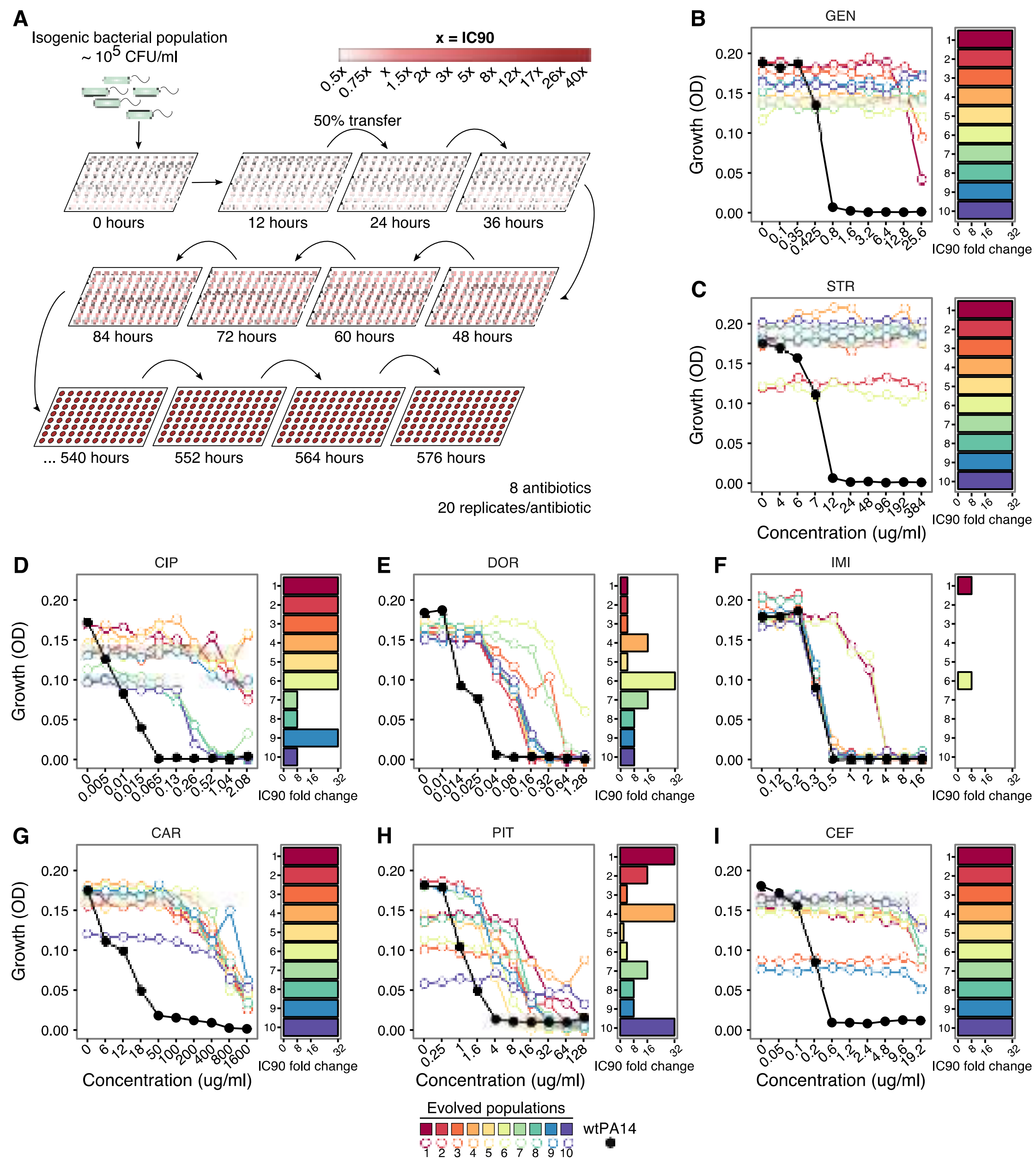


Fig. 1. Directional selection of highly resistant *P. aeruginosa*. *(A)* Illustration of the experimental design used for the selection of resistant populations. Twenty replicate populations for each of the eight included antibiotics (Table 1) and a control without antibiotic (a total of 180 populations) were serially transferred every 12h into fresh medium and, for the drug treatments, increasing concentrations of each drug. Selection was initiated at 0.5 times the concentration inhibiting >90% of growth (IC90) and concluded at ~40 times of the IC90. *(B-I)* Ten populations for each antibiotic were subsequently evaluated for their growth on different concentrations of the drug experienced during the experiment. Dose-response curves are shown in the left panels and IC90 fold changes in the right panels. The ten replicate populations are shown in different colors, while the black line represents the ancestral *P. aeruginosa* PA14.

170

171 **Rapid resistance evolution entails a growth cost**

172 To evaluate whether the evolution of high drug resistance levels was generally
173 associated with a fitness deficiency, we measured different growth parameters of all of
174 the evolved populations, and the ancestral PA14 in a drug-free environment. We found
175 that the control populations, which evolved without antibiotics, had significantly
176 shorter lag phases and lower growth rates relative to the ancestor (supplementary fig.
177 S1). These results suggest that adaptation to the medium alone can have strong effects
178 on fitness. These effects could in turn influence antibiotic resistance. To evaluate this,
179 we compared the sensitivity of the evolved control populations and the ancestor against
180 each of the eight antibiotics. In general, we found no significant differences between
181 the dose response curves of the controls and the ancestor (supplementary fig. S2). The
182 only exceptions refer to cases at sub-inhibitory drug concentrations where the evolved
183 populations showed higher growth than the ancestral PA14. More importantly, with
184 only a single exception, none of the replicate populations showed a change in the IC90,
185 thus indicating that the fitness alterations of the evolved controls do not translate into
186 changes in drug sensitivity (supplementary fig. S2).

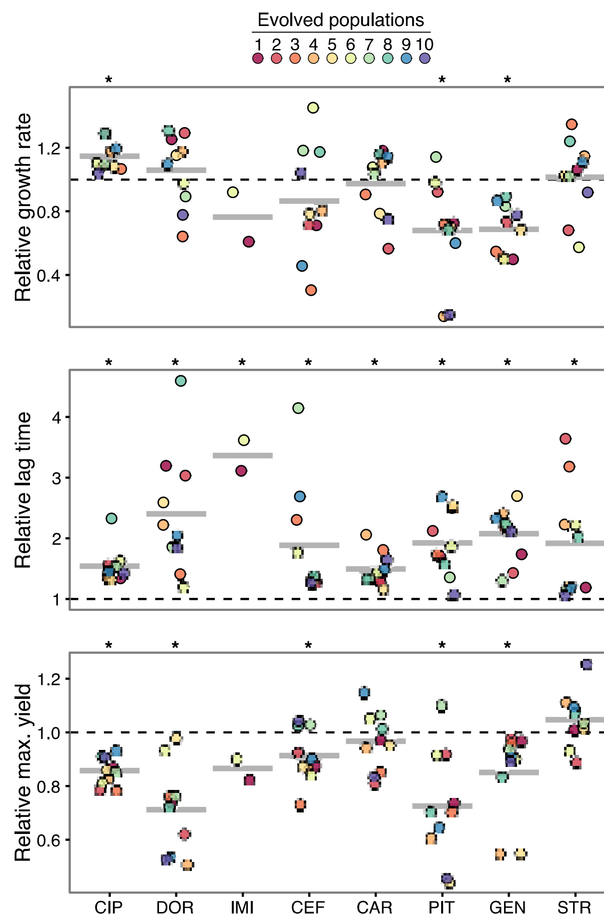
187

188 As evolution in the absence of drugs affected growth characteristics, we specifically
189 assessed the costs associated with the adaptation to antibiotics by relating our growth
190 measures of the 80 resistant populations to those of the evolved controls. Resistant
191 populations showed changes in at least one of the measured growth characteristics: lag
192 time, growth rate, and maximum yield (fig. 2). In all cases, we observed a significantly
193 longer time spent in lag phase, and in most cases (5 out of 8 drugs), a significant
194 reduction of about 20-50% in maximum yield. Only populations adapted to PIT and
195 GEN had significantly reduced growth rates. Overall, 90% of the resistant populations
196 spent longer times in lag phase, 49% produced lower maximum yields, and 36% had
197 lower growth rates (supplementary table S1). Interestingly, 60% of the resistant
198 populations had a significant cost in at least two of the parameters, whereas only five
199 of the populations showed no fitness costs at all.

200

201 In most cases there was substantial variation among populations adapted to the same
202 drug (fig. 2). This might have been the result of at least two paths: a direct outcome of
203 costly adaptation resulting in varying lengths of lag phases depending on the favored

204 resistance mechanism, or, alternatively, an indirect consequence of our experimental
205 design in which selection independently acted in favor of tolerance. In the latter case,
206 an extended lag phase could have allowed *P. aeruginosa* to remain in the environment
207 without immediately changing its initial resistance levels but instead increasing
208 antibiotic tolerance. This could have ultimately resulted in varying levels of measured
209 resistance among the various populations (Levin-Reisman et al. 2017). However, we
210 found no significant correlation between the IC90 fold change and the lag time (or any
211 of the growth parameters) for any of the antibiotics used (supplementary table S2 and
212 supplementary fig. S3). This strongly suggests that the differences in resistance are not
213 a result of selection for tolerance, but rather due to underlying differences in the
214 resistance mechanisms that in turn have distinct effects on growth. This finding also
215 means that the extent of the resistance increase does not linearly translate into a fitness
216 cost, but most likely depends on the specific underlying mechanism.
217

218 

219

220 **Fig. 2.** Relative fitness in the absence of antibiotics. Shown, from top to bottom, is
221 fitness relative to the average of the evolved control populations, calculated for growth

222 rate, lag time, and maximum yield for all populations adapted to one of the eight
223 antibiotics (X-axis) after 24h of growth in antibiotic-free media. Colored points
224 represent the replicate populations and the horizontal grey crossbars indicate the mean
225 for each antibiotic. Black dashed lines highlight equality to the controls; values above
226 indicate a fitness advantage whereas values below denote a cost. Plase note that for the
227 lag phase, this is inverted: values larger than 1 indicate a longer time spent in lag phase
228 and thus a fitness cost. Asterisks on top of each panel indicate significant difference
229 from 1 (i.e., a significant change in fitness) using a Wilcoxon Rank test with probability
230 adjustment based on the false discovery rate (FDR) to account for multiple testing.
231
232
233 The extent to which growth costs are associated with antibiotic resistance evolution has
234 been evaluated for a variety of combinations of bacteria and drugs, including *P.*
235 *aeruginosa* (Kugelberg et al. 2005; Andersson and Hughes 2010; Melnyk et al. 2015).
236 These studies demonstrated that mutations conferring high levels of resistance usually
237 lead to larger fitness costs. Moreover, after adaptation to increasing drug
238 concentrations, a higher number and more complex types of mutations (i.e., large
239 deletions) coincide with larger defects in growth rate (Lázár et al. 2014). Considering
240 such substantial resistance costs, it is surprising that resistant mutants persist both in
241 clinical and experimental environments. Several factors were previously found to
242 ameliorate or completely alleviate the effects of resistance mutations on growth. For
243 instance, quinolone resistant *P. aeruginosa* showed a high prevalence of cost-free
244 mutations as well as the emergence of compensatory mutations in genes that,
245 unexpectedly, are not directly associated to the fitness defect (Kugelberg et al. 2005).
246 In fact, some of our populations showed an increase in fitness in some or all of the
247 growth parameters measured, emphasizing that the costs associated with some resistant
248 mechanisms can be ameliorated rapidly (within 58 generations) or incur no costs at all.
249 Furthermore, the role of epistatic effects, the genetic background in which mutations
250 occur, and the environment also seem to play an important role in the magnitude of the
251 cost (Melnyk et al. 2015). Therefore, there is a lack of predictability, constraining the
252 potential clinical use of such information for a rational design of treatment strategies.
253
254 **Comprehensive collateral effects upon antibiotic resistance evolution**

We then tested the evolved populations for the presence of collateral sensitivity or resistance against all other drugs. For this we challenged each population against various concentrations (in triplicate) of all the antibiotics and compared their growth to the ancestral PA14. To quantify the change in resistance or sensitivity we considered growth relative to the no-drug environment, which accounts for general fitness changes resulting from adaptation to the experimental environment. We then calculated the area under the curve (AUC) of the ancestral PA14 and subtracted it from that of each population (fig. 3A and B, for the IC90 fold changes see supplementary fig. S4). Finally, we counted how often adaptation to one antibiotic led to resistance against any other, hereby defined as direct adaptation, and also; how many times resistance to a given drug evolved after adaptation to another one, hereby defined as indirect adaptation (fig. 3C). The same process was repeated to calculate the number of cases of sensitivity by direct or indirect adaptation.

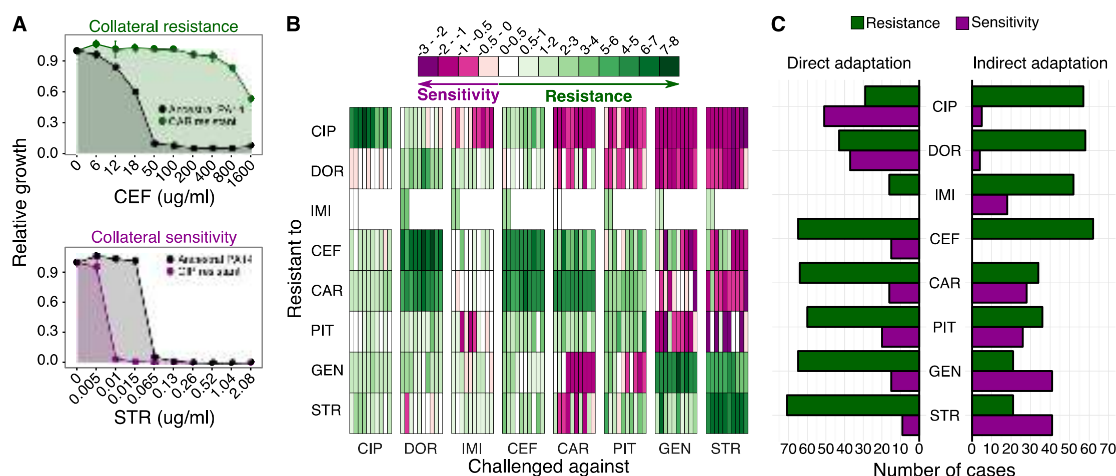
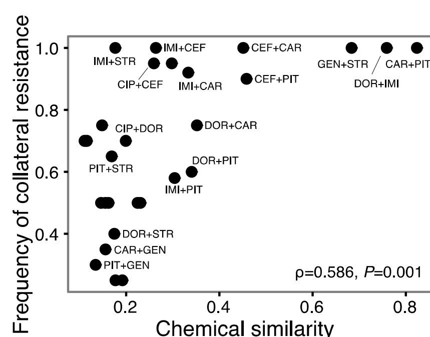


Fig. 3. Evolution of collateral sensitivity and resistance. *(A)* Illustration of the quantification of evolved collateral resistance or sensitivity. Bacterial growth (relative to a no-drug environment) of all evolved populations and the ancestral *P. aeruginosa* PA14 is first measured across concentrations of the various antibiotics. The area under the curve of the ancestor is subsequently subtracted from that of each population; resulting negative values indicate sensitivity (bottom panel), whereas positive values denote resistance (top panel). *(B)* The collateral profiles of all experimentally evolved populations (rows refer to the drugs used during experimental evolution), challenged against all other antibiotics (as indicated by columns). The vertical bars within each

281 block represent the replicate population. The different shades of purple or green
282 highlight the extent of sensitivity or resistance, respectively. *(C)* We counted the total
283 number of cases, for which adaptation to a particular antibiotic (listed in the middle)
284 led to collateral sensitivity or resistance (direct adaptation; left panel), and also the total
285 number of cases, for which sensitivity or resistance towards the focal antibiotic was
286 observed upon adaptation to any of the other drugs (indirect adaptation; right panel).
287
288 Overall, we identified collateral effects in almost all population with evolved
289 resistances, whereby cross-resistance is generally more common than collateral
290 sensitivity (supplementary fig. S5 and fig. 3B and C; direct adaptation). Essentially
291 identical results are obtained if IC90 fold change is used for the analysis rather than
292 AUC differences (supplementary fig. S4). Five main conclusions can be drawn from
293 our analysis. (i) Adaptation to a given antibiotic leads to collateral resistance to drugs
294 of similar classes (see the aminoglycosides STR, and GEN, or the β-lactams CEF, PIT
295 and CAR). (ii) Resistance to most drugs leads to collateral sensitivity against
296 aminoglycosides, whereas resistance to aminoglycosides leads to collateral sensitivity
297 almost exclusively to the penicillin-type β-lactams (PIT and CAR; fig. 3B and C;
298 indirect adaptation). (iii) Adaptation to CIP or DOR leads to enhanced sensitivity
299 against most other drugs, but interestingly this effect appears to be unidirectional:
300 adaptation to other antibiotics rarely leads to sensitivity against these two drugs (fig.
301 3C; indirect resistance). (iv) Collateral resistance against CEF, a 3rd generation
302 cephalosporin with specific activity against *P. aeruginosa*, is found upon resistance
303 evolution towards all other drugs, suggesting that resistance to some cephalosporins
304 may be readily achieved indirectly, thus compromising its use as a second-line drug.
305 (v) In several cases, substantial variation was observed among populations adapted to
306 the same drug, suggesting that different resistance mechanisms may lead to contrasting
307 patterns of collateral effects.
308
309 The evolution of collateral resistance and sensitivity seems to be a widespread trade-
310 off in bacteria following drug resistance evolution. For example, in *E. coli*, collateral
311 resistance towards drugs of the same class was repeatedly observed (Imamovic and
312 Sommer 2013; Lázár et al. 2013; Lázár et al. 2014; Oz et al. 2014). These previous
313 studies also revealed interesting exceptions. Some drugs, such as CIP, were often
314 targets of indirect multi-drug resistance evolution, regardless of the similarity of the

315 drug used during experimental evolution. Also, the strength of cross-resistance was
316 variable even among drugs with the same cellular targets. For instance, within the cell-
317 wall inhibitors, adaptation to penicillins seems to lead to cross-resistance more often
318 than adaptation to carbapenems.
319
320 It is noteworthy that the direction of collateral sensitivity differs in specific cases
321 between *P. aeruginosa* and *E. coli*. In particular, resistance to the aminoglycosides
322 often preceded a two-fold reduction in MIC against most other drugs in *E. coli*
323 (Imamovic and Sommer 2013; Lázár et al. 2013). In contrast, adaptation to the same
324 drugs in PA14 more often led to cross-resistance, while in some individual populations
325 collateral sensitivity was observed almost exclusively towards the penicillins.
326 Moreover, CIP-adapted *E. coli* strains usually exhibited cross-resistance to most other
327 drugs (Imamovic and Sommer 2013; Lázár et al. 2014), while our experiments with *P.*
328 *aeruginosa* produced hypersensitivity in such combinations. These findings highlight
329 the presence of key differences between species in the evolved collateral effects
330 following drug adaptation. Systematic evaluation of different pathogen-drug
331 combinations is thus essential for a full appreciation of incidences and diversity of
332 evolved collateral sensitivity (Imamovic and Sommer 2013; Lázár et al. 2014).
333
334 Unexpectedly, our results further demonstrate the presence of substantial variation in
335 collateral effects upon adaptation to one drug. In several cases, we even observed
336 contrasting patterns of either collateral sensitivity or resistance. For example, CEF-
337 adapted populations exposed to GEN produced three cases of cross-resistance, five
338 cases of collateral sensitivity, and two neutral effects (fig. 3A; fig. S4). Similar variation
339 in collateral effects are also found for all other antibiotic treatments, with the exception
340 of IMI, most likely due to low sample size (i.e., only 2 populations could be included
341 in the analysis). Moreover, such divergence in collateral effects upon antibiotic
342 resistance evolution has not yet been reported for other bacterial species. These findings
343 strongly suggest that our replicate *P. aeruginosa* populations from a particular
344 antibiotic treatment achieved resistance through different molecular mechanisms,
345 which in turn had opposite effects on the interaction with a second antibiotic.
346
347 **Cross-resistance may result from the chemical similarity between drugs**

348 Recent studies have followed Szybalski and Bryson's hypothesis that the chemical
349 relatedness between different drugs could explain the prevalence of cross-resistance,
350 finding a partial correlation between them in *E. coli* (Lázár et al. 2014). However, the
351 reported relationship was strongly biased by the aminoglycosides, since no cross-
352 resistance to such drugs was observed and these possess a chemical structure that is
353 fundamentally different to that of the other drugs. In the case of *P. aeruginosa*, we often
354 observed cross-resistance to the aminoglycosides, thus allowing us to further explore
355 the range of relationships between drug similarities and the frequency of cross-
356 resistances. For such an analysis, we first inferred the chemical similarity among all
357 antibiotics by calculating the Jaccard's index obtained from the pairwise comparison of
358 their chemical fingerprints (supplementary fig. S6). We then related these similarities
359 to the frequencies of collateral resistances (FCR) for all drug pairs (see Materials and
360 Methods, and Lázár et al. 2014). Our analysis revealed a significant correlation between
361 these two parameters (fig. 4). Importantly, drugs targeting the same cellular process are
362 not necessarily chemically similar (e.g., IMI and PIT or DOR and CAR) and in these
363 cases we do not find high levels of cross-resistance. Altogether, chemical similarity
364 appears in most cases to be a key determinant of the probability of cross-resistance.
365

366 

367

368 **Fig. 4.** Chemical similarity correlates with frequency of collateral resistance. Pairwise
369 Jaccard's similarity indexes where calculated based on the chemical fingerprints of
370 each antibiotic. The frequency of collateral resistance (FCR) was then determined as
371 $FCR = \left(R_{A\to B} + R_{B\to A}\right) / L_{AB}$, where $R_{A\to B}$ is the number of populations resistant to drug
372 A with cross-resistance to drug B (and *vice versa* for $R_{B\to A}$), and L_{AB} is the total number
373 of populations adapted to A and B. A significant correlation was then found between
374 the chemical similarity and the FCR (Spearman's rank correlation). Each point

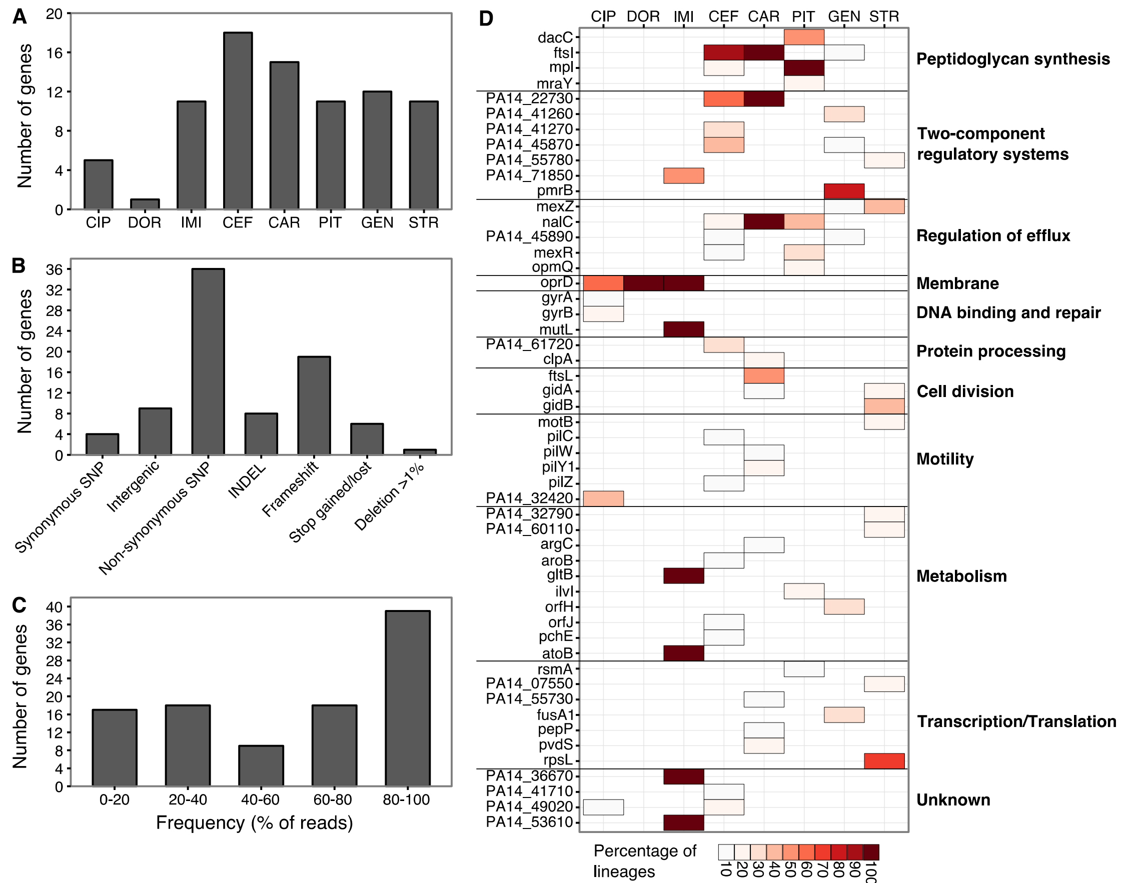
375 corresponds to a chemical comparison between any two given drugs. Labels are shown
376 for some, but not all, of these pairwise comparisons.
377

378 **Distinct mutations underlie the evolution of high antibiotic resistance**

379 To better understand the genetic mechanisms selected during the evolution of high
380 antibiotic resistance, we obtained whole genome sequences for 81 evolved populations:
381 71 antibiotic adapted populations and 10 controls. We characterized genomic variations
382 using a previously established analysis pipeline (see Materials and Methods; Jansen et
383 al. 2015). An important step of the pipeline is to remove substitutions which occurred
384 in the control populations, as these may result from adaptation to general experimental
385 conditions and could thus obscure the signals relevant for adaptation to the antibiotics
386 (see supplementary table S4 for a list of mutations found in the evolved control
387 populations). We further annotated the genes with mutations in coding regions using
388 DAVID, the *Pseudomonas* Database (available online at: http://pseudomonas.com),
389 and published information to group them by function and their likely involvement in
390 antibiotic resistance. In general, we observed an average of 10.5 genes affected per
391 antibiotic environment (fig. 5A), but with different degrees of mutational diversity
392 (supplementary fig. S7). Most mutations were non-silent and may thus have contributed
393 to adaptation (fig. 5B and C): 89% of the mutations were observed in coding regions
394 (Intergenic vs. all other mutations, χ^2 test, $\chi^2 = 50.94$, df = 1, $P < 0.0001$); 83% of the
395 variants found in coding regions (e.g., different types of non-synonymous,
396 insertions/deletions [indels], or frameshift mutations) led to partial or complete loss of
397 function (Intergenic and Synonymous SNPs vs. all other type of variants, χ^2 test, $\chi^2 =$
398 39.15, df = 1, $P < 0.0001$), and more than half of the variants observed were fixed at
399 levels above 40% (Number of variants <40% fixed vs. variants ≥40% fixed, χ^2 test, χ^2
400 $= 50.94$, df = 1, $P = 0.003$).
401

402 For subsequent analysis we focused on genes having mutations in their coding regions
403 only (fig. 5D). Interestingly, the only two surviving populations adapted to IMI showed
404 mutations in *mutL,* a gene coding for a DNA repair enzyme generally associated with
405 hypermutator phenotypes (Oliver et al. 2002; Montanari et al. 2007; Ciofu et al. 2010);
406 these populations accumulated as many mutations in half the number of generations as
407 the others during the entire experiment (supplementary table S5). The various DOR

408 adapted populations produced a unique SNP in a single gene, *oprD*: a membrane protein
409 (fig. 5D). The populations adapted to all other antibiotics showed a larger number of
410 genomic changes across a variety of genes (fig. 5D).
411



412
413 **Fig. 5.** Genomics of adaptation. Distribution of the number of genes with substitutions
414 per *(A)* antibiotic used in the evolution experiment, (B) type of mutational change, and
415 (C) average frequency class within the replicate populations. (D) Functional effect of
416 mutations found in coding regions of the listed genes (vertical axis, left side) across
417 evolution experiments with different antibiotics (horizontal axis). Functional
418 information (right side) is inferred from a combined analysis using DAVID, the
419 Pseudomonas database and publications. Different shades of red indicate the percentage
420 of affected populations per evolution experiment with a particular antibiotic.
421
422 The functional annotation revealed changes in three main functions across evolved
423 populations (fig. 5D): The first group of affected genes is related to direct targets of the
424 β-lactam antibiotics and was primarily identified in populations adapted to CEF, CAR
425 and PIT. This group included genes associated with peptidoglycan synthesis

426 (supplementary table S4): the penicillin-binding protein 3 (PBP3) *ftsI*, and also the
427 UDP-N-acetylmuramate: L-alanyl-gamma-D-glutamyl-meso-diaminopimelate ligase
428 *mpI*. Non-silent mutations in these genes were previously demonstrated to increase
429 resistance against β-lactams, either by limiting the interaction between antibiotics and
430 the products of these genes, or by indirectly inducing SOS responses or enhanced efflux
431 (Miller et al. 2004; Tsutsumi et al. 2013).
432
433 The second group of genes is related to 7 different two-component regulatory systems
434 (fig. 5D). This group was generally affected in response to adaptation to β-lactam and
435 aminoglycoside antibiotics; whereby variation in mutated systems seem to depend on
436 the antibiotic class. Two-component systems are essential for bacteria to recognize
437 different environmental stimuli and coordinate a fine-tuned response via a complex
438 phosphorylation signal transduction system (Hoch 2000; Robinson et al. 2000; Ramos
439 et al. 2005). *P. aeruginosa* in particular possesses a large number of such regulatory
440 systems, which it uses to control cellular division, development, stress responses, and
441 pathogenicity (Rodrigue et al. 2000).
442
443 The third main group of mutated genes was found in β-lactam and aminoglycoside
444 adapted populations, and included different efflux regulatory systems (fig. 5D). These
445 genes modulate the activity of distinct efflux pumps which can lead to single or multi-
446 drug resistance (Hancock and Speert 2000; Li et al. 2000; Poole 2001; Piddock 2006).
447 Importantly, two of them (*mexZ* and *nalC*) are closely related to those regulating the *tet*
448 efflux pumps (TetR-like repressors), which were previously demonstrated to contribute
449 to collateral sensitivity against aminoglycosides in tetracycline resistant *E. coli*
450 (reviewed in Baym, Stone, et al. 2016). These genes were further proposed as a pivotal
451 group to be exploited in sequential treatment strategies (Baym, Stone, et al. 2016).
452
453 We additionally identified changes in several other known or at least suggested
454 antibiotic resistance genes (fig. 5D), including the membrane protein main component
455 *oprD*, the DNA gyrase subunits *gyrA* and *gyrB*, as well as other genes involved in
456 cellular processes such as cell division and motility (Macfarlane et al. 2000; Drenkard
457 and Ausubel 2002; Livermore 2002; Amin et al. 2005). Our analysis further revealed
458 changes in genes with currently unknown function and/or no previous implication in
459 antibiotic resistance, and/or mutations in non-coding regions, which may still influence

460 resistance if regulatory regions are affected (e.g., possibly relevant for DOR-adapted
461 populations). Some of these changes occurred at lower frequency within the replicate
462 populations, but their exact contribution to the observed evolutionary pattern is
463 uncertain and may be interesting to address in future studies. Furthermore, among the
464 PIT-adapted populations we also found substantial variation in the sets of mutated
465 genes, even though they almost all consistently affected the same two functional
466 categories: peptidoglycan synthesis and efflux regulatory systems. This variation could
467 explain the different levels of resistance observed for these populations, whereby
468 different groups of mutated genes lead to higher or lower levels of resistance.
469

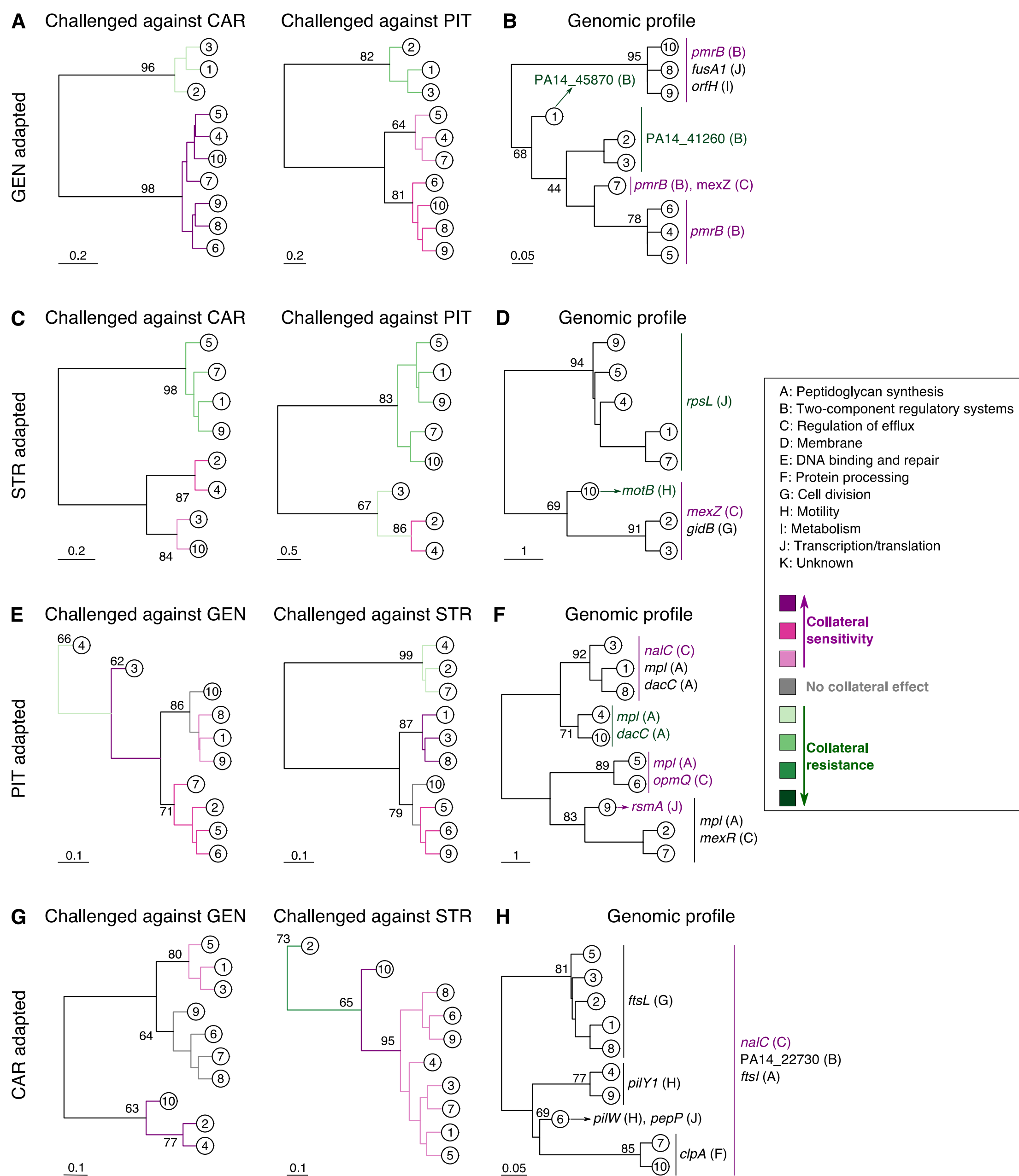
470 **Specific resistance mechanism associate with high growth costs**

471 The accumulation of multiple mutations during adaptation to antibiotics could translate
472 into stronger reductions in fitness under drug-free conditions. In fact, in a similar study
473 with *E. coli*, populations accumulating a large number of mutations or deletions had
474 also very low fitness in drug-free environments (Lázár et al. 2014). Similarly, in *P.*
475 *aeruginosa* we found that the populations with the strongest decrease in growth rate
476 have a significantly larger number of mutations in their genomes (supplementary fig.
477 S8A). However, none of the other measured fitness parameters correlated with the
478 number of mutations, suggesting that this alone is not enough to explain the observed
479 variation in fitness. Moreover, when we looked for overrepresented genes in
480 populations with the most extreme growth costs, we observed some functions to be
481 more prevalent than others (supplementary fig. S8B-D). In particular, genes involved
482 in peptidoglycan synthesis (*mpl*, *dacC* or *ftsI*), regulation of efflux (*nalC*) or part of
483 two-component regulatory systems (*pmrB*) were more frequently found in populations
484 with lower relative fitness across the measured growth parameters. Interestingly,
485 multiple genes affecting these cellular processes were found within the same
486 populations having strong fitness reductions (*nalC* together with *mpl* and *dacC* were
487 often found in PIT resistant populations). Altogether, the costs of adaptation seem to
488 be dependent on both the number of mutations accumulated and the specific mutated
489 resistance mechanism.
490

491 **Parallel patterns of collateral resistance across treatments is not linked to similar**
492 **mutational profiles**

493 Next, we assessed whether parallel mutational changes could explain the observed
494 patterns of collateral resistance across different treatments in *P. aeruginosa* (Fig. 3B).
495 In contrast to the findings for *E. coli* (Lázár et al. 2014), we found comparatively little
496 mutational overlap between the populations from different antibiotic treatments
497 (supplementary fig. S9A). Interestingly, in the few cases where similar mutational
498 profiles were observed among populations resistant to different drugs, the two
499 compared antibiotics appear to be chemically related, and the populations showed
500 cross-resistance (supplementary fig. S9B and C). However, these correlations were not
501 statistically significant, because our data set also included several populations with high
502 cross-resistance against chemically similar drugs, but with completely distinct
503 mutational profiles (supplementary fig. S9B and C). Therefore, it seems that the parallel
504 patterns of cross-resistance across treatments are based on different sets of mutations.
505
506 This is also true for populations from different treatments that adapted to drugs of the
507 same class. For instance, the populations that adapted to the two aminoglycosides
508 showed high levels of cross-resistance among them, but adaptation to GEN was more
509 often accompanied by mutations in the two-component regulatory gene *pmrB,* while
510 those adapted to STR predominantly showed mutations in genes involved in cellular
511 division (i.e., *gidA* or *gidB*), or in efflux regulation (i.e., *mexZ*; fig. 5D and
512 supplementary table S5).
513
514 **Contrasting collateral effects have unique genomic profiles**
515 We identified substantial variation in the incidence of collateral sensitivity and cross-
516 resistance within particular evolution experiments. For instance, five of the CEF
517 adapted populations showed collateral sensitivity against GEN and STR, whereas the
518 rest suffered cross-resistance or neutral effects against the same drugs (fig. 3B).
519 Similarly, three of the GEN adapted populations displayed collateral sensitivity against
520 CAR and PIT, while the others were resistant (fig. 3B). Such variation could be the
521 result of distinct resistance mechanisms selected during adaptation to each drug, which
522 in turn led to contrasting sensitivities against other antibiotics. To explore this
523 possibility, we focused on four exemplary cases, for each of which we repeatedly found
524 contrasting variation in collateral effects to other antibiotics. These included the
525 populations adapted to the two aminoglycosides (GEN, STR), which both showed such
526 variation towards PIT and CAR (Fig. 3B). We also considered the reverse two cases,

527 for which populations that had adapted to either PIT or CAR produced contrasting
528 collateral patterns towards GEN and STR (Fig. 3B). For these four cases, we used
529 hierarchical cluster analysis (based on Ward's criterion method and bootstrapping to
530 assess cluster stability (Murtagh and Legendre 2014)) to relate populations with varying
531 collateral effects to corresponding genomic variation. In particular, we first assessed
532 whether populations adapted to a given drug would cluster together based on their
533 sensitivity against the two other antibiotics (fig. 6A, 6C, 6E, and 6G). Then, we asked
534 whether the same populations would also cluster together based on their sets of mutated
535 genes (fig. 6B, 6D, 6F, and 6H).
536

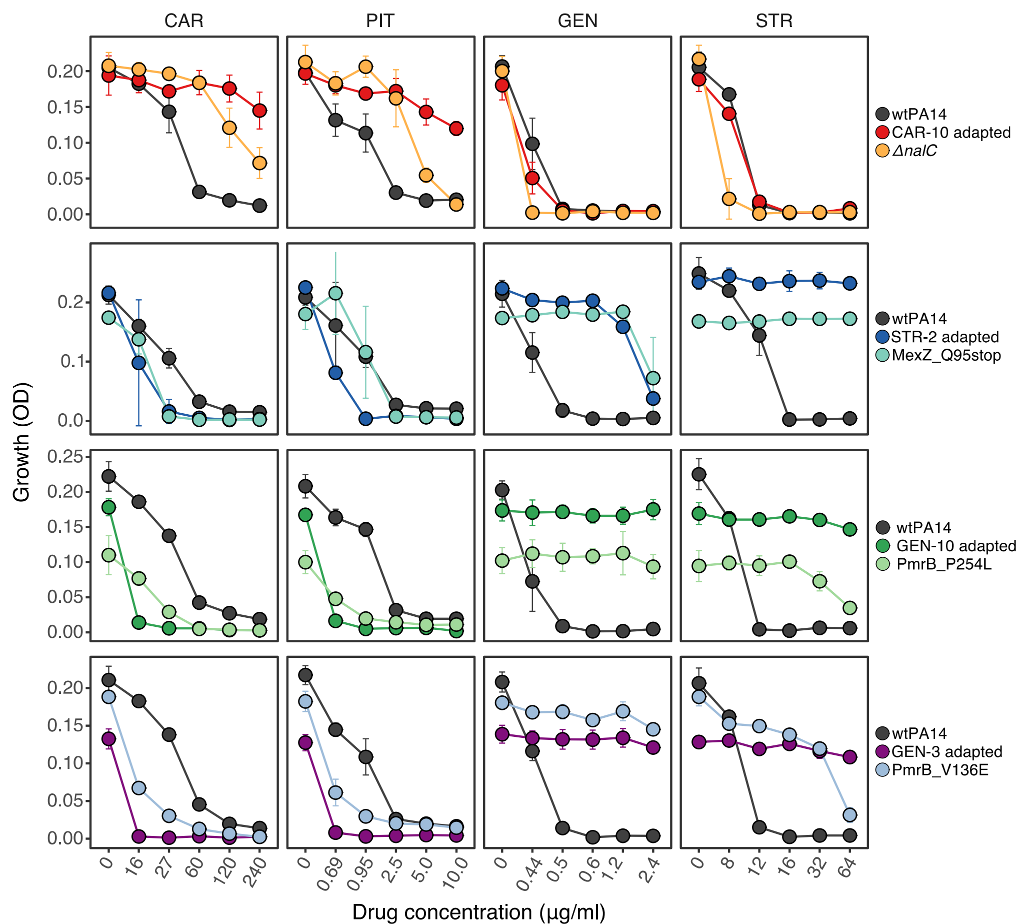


537

538 **Fig. 6.** Genomic determinants of collateral sensitivity. We employed a hierarchical
539 clustering analysis using the Ward's criterion method and bootstrapping to identify the
540 genomic determinants of variation in collateral profiles in four treatments. In two of
541 these treatments, replicate populations that had adapted to either GEN (A,B) or STR
542 (C,D) produced variation in collateral profile to PIT and CAR. In the other two
543 treatments, replicate populations that had adapted to either PIT (E,F) or CAR (G,H)
544 showed such variation towards GEN and STR. We first evaluated the clustering of
545 populations adapted to GEN (A), STR (C), PIT (E) and CAR (G) based on the strength
546 of collateral effects to the other two drugs (Fig. 3B), highlighting clusters of those with
547 collateral sensitivity (lines in different shades of purple, see legend to the right) versus
548 those with collateral resistance (lines in different shades of grey). The circled numbers
549 always indicate the same replicate populations from a particular treatment across the
550 related panels. Populations clustering together based on their collateral effects also
551 often clustered together based on their genomic profile (B, D, F, H). The mutated genes
552 present in the various clusters are given to the right of the dendrograms, followed by
553 letters for their functional annotation in brackets (see legend to the right for the
554 annotation categories). If a specific cluster mainly included populations associated with
555 collateral sensitivity, then the gene names are given in purple. In cases, where clusters
556 mainly included populations associated with collateral resistance, the gene name is
557 given in green.
558
559 We identified clusters that consistently link the contrasting patterns of collateral
560 sensitivity to particular genomic variations. Intriguingly, the two-component regulatory
561 system and TetR-like repressors appear to play a pivotal role in determining these
562 patterns. For the GEN-adapted populations, sensitivity to PIT and CAR was associated
563 with mutations in *pmrB* (fig. 6A and 6B), a sensor kinase implicated in resistance
564 against cationic antimicrobial peptides, polymyxins, and aminoglycosides in
565 *Salmonella* and *P. aeruginosa* (McPhee et al. 2003). Aminoglycoside resistance
566 mediated by *pmrB* is hierarchically controlled by two regulatory systems (PhoP-PhoQ
567 and PmrB-PmrA) that ultimately remodel lipid A in the outer membrane resulting in a
568 reduction of the membrane's negative charge. Such alterations were shown to enhance
569 sensitivity to β-lactams in aminoglycoside resistant *E. coli* (Lázár et al. 2013; Baym,
570 Stone, et al. 2016). Altogether, this suggests that *pmrB* could be the main driver of β-
571 lactam sensitivity in GEN resistant populations.

572
573 Collateral sensitivity against penicillins in STR-adapted populations is linked to
574 mutations in the gene *mexZ* (fig. 6C and D), a TetR-like repressor modulating the
575 expression of the MexXY-OprM efflux system (Aires et al. 1999). This gene was found
576 to mediate aminoglycoside resistance in *P. aeruginosa* PAO1 and clinical isolates from
577 cystic fibrosis patients by increasing cellular drug efflux, trough an up-regulation of the
578 *mexXY* genes (Westbrock-Wadman et al. 1999). Interestingly, in *P. aeruginosa*,
579 extrusion of the aminoglycosides appears to be exclusively mediated by MexXY-
580 OprM, which, when overexpressed, is also able to extrude most penicillins (except for
581 carbenicillin and sulbenicillin), some cephalosporins, and meropenem (Masuda et al.
582 2000). However, to the best of our knowledge, its possible role in mediating collateral
583 sensitivity against penicillins has not been reported before. In addition, the expression
584 of collateral sensitivity could be further influenced by other mutations. For instance, a
585 mutation in *motB*, a gene required for flagellar motility in *P. aeruginosa* (Doyle et al.
586 2004), is present in addition to mutated *mexZ* in population 10, which shows collateral
587 sensitivity to CAR but not PIT (fig. 6C and 6D).
588
589 In the case of the penicillin-adapted populations, collateral sensitivity against
590 aminoglycosides appears to be associated – among others – with mutations in the gene
591 *nalC*, an efflux regulator of the MexAB-OprM system known to confer intermediate to
592 high levels of resistance to multiple drugs (Cao et al. 2004). Populations adapted to
593 CAR and PIT with mutations in this gene consistently showed a more sensitive
594 phenotype against both aminoglycosides (fig. 6E-H). Interestingly, *tet* efflux regulators,
595 which are related in their function to *nalC*, were previously shown to mediate collateral
596 sensitivity against aminoglycosides in tetracycline resistant *E. coli* (reviewed in Baym,
597 Stone, et al. 2016), thus suggesting a pivotal role of this type of resistance mechanism
598 in determining antibiotic susceptibility trade-offs.
599
600 It is noteworthy that the exact distribution of the variation in sensitivity against
601 aminoglycosides in CAR-adapted populations is not well captured by our approach. All
602 the identified clusters show genomic changes that affect the same three functions, yet
603 the exact mutations and their frequencies within the populations differ (fig. 6G and H,
604 supplementary fig. S10 and supplementary table S5). The observed phenotypic
605 variation may then be caused by only some of the variable genes, by specific mutations

606 only, by the combination of specific allelic variants, or by the frequency difference of
607 certain mutations. Therefore, the observed collateral effects are likely influenced by
608 additional factors, which could not be identified directly by the cluster analysis.
609
610 **Mutations in *nalC*, *mexZ*, and *pmrB* can cause collateral sensitivity**
611 Different regulatory systems were generally associated with collateral sensitivities in
612 the evaluated populations. To further evaluate such a role, we assessed antibiotic
613 sensitivity in genetically modified ancestral PA14, in which we re-introduced a
614 selection of four of the identified mutational changes (Materials and methods), namely
615 the inferred ~500 bp deletion in *nalC*, a SNP in *mexZ* leading to an early stop codon
616 (Q95stop), and two non-synonymous mutations in *pmrB* causing a P254L and a V136E
617 amino acid substitution. In all cases, the constructed mutants showed almost the exact
618 same response as the evolved populations against at least two of the considered
619 antibiotics (fig. 7 and supplementary table S6). In particular, the SNPs introduced in
620 *pmrB* and *mexZ*, originally observed in the GEN- and STR-adapted populations, caused
621 high resistance to these aminoglycosides, and led to significantly increased sensitivity
622 to the penicillins PIT and CAR (fig. 7; supplementary table S6). Similarly, the
623 introduced deletion in *nalC*, originally observed in CAR-adapted populations, caused
624 resistance to the tested penicillins while significantly increasing sensitivity to both GEN
625 and STR (fig. 7; supplementary table S6). Thus, two-component regulatory systems
626 and efflux regulators can be responsible for the observed collateral sensitivity in *P.*
627 *aeruginosa*.
628



629

630 **Fig. 7**. Functional analysis of different regulatory genes. We focused on four specific
631 mutations identified in the evolved populations to associate with collateral sensitivity
632 and introduced these into the ancestral PA14 strain. The resulting mutants were then
633 tested against various concentrations of CAR, PIT, GEN and CAR (from left to right).
634 In all cases, the ancestral PA14 (always in black) and the adapted population (always
635 in darker colors), from which the particular mutation was extracted, were tested
636 simultaneously with the corresponding constructed mutants. Points and error bars show
637 the mean OD ± SD of 5 technical replicates per antibiotic concentration. For each set
638 of bacterial populations challenged against a particular drug we performed a GLM
639 followed by Tukey's honest significant difference (HSD) test. For a summary of the
640 statistical results see supplementary table S5.

641

642 The tested genes *nalC*, *mexZ*, and *pmrB* are involved in the regulation of efflux pumps
643 or alteration of the outer membrane, and can thus influence antibiotic resistance
644 (Westbrock-Wadman et al. 1999; McPhee et al. 2003; Cao et al. 2004; Daigle et al.
645 2007; Lázár et al. 2013; Baym, Stone, et al. 2016). To the best of our knowledge, the
646 results of our functional genetic analysis demonstrated for the first time in *P.*

647 *aeruginosa* that such genes can also differentially lead to collateral sensitivity against
648 aminoglycosides (*nalC*) and penicillins (*mexZ* and *pmrB*). Importantly, two distinct
649 aminoglycoside resistance mechanisms independently led to penicillin
650 hypersensitivity: a loss of function (LOF) mutation in *mexZ,* and two different non-
651 synonymous mutations in the two-component regulatory system gene *pmrB*.
652 Intriguingly, contrasting collateral effects emerged from alterations in functionally
653 related genes: both *mexZ* and *nalC* are from the TetR-family of repressors, and LOF
654 mutations in each of them led to completely opposite susceptibilities against penicillins
655 and aminoglycosides. Such contrasting results could be due to an impairment of one of
656 the efflux pumps upon up-regulation of the other, a phenomenon previously seen in
657 other efflux systems such as MexCD and MexAB, or MexEF and MexAB (Gotoh et al.
658 1998; Maseda et al. 2000; Jeannot et al. 2008), but not between MexXY and MexAB.
659

660 **Conclusions**

661

662 We here demonstrated that *P. aeruginosa* adapts rapidly to high-level antibiotic stress
663 and that such adaptation may influence resistance against other antibiotics. Cross-
664 resistance correlates well with chemical similarity of the antibiotics, in agreement with
665 previous work. Collateral sensitivity was identified in several cases, yet different in
666 direction to those cases previously reported for other bacteria, such as *E. coli*.
667 Surprisingly, adaptation to a particular antibiotic produced both cross-resistance and
668 collateral sensitivity across the replicate populations. Our genomic analysis suggests
669 that alternative mechanisms were favored during resistance evolution, which then
670 resulted in these contrasting cross-resistance patterns. Intriguingly, regulatory systems
671 appear to play a key role in mediating the observed collateral effects. Functional genetic
672 analysis revealed that four of the identified mutations in three regulatory genes (*nalC*,
673 *mexZ*, and *pmrB*) can indeed cause collateral sensitivities in *P. aeruginosa*. Overall, we
674 expect our results to help the development of novel antibiotic therapy that exploits
675 fitness trade-offs during drug resistance evolution.
676
677

678 **Materials and methods**

679 **Bacteria and media**

680 All experiments were conducted with *Pseudomonas aeruginosa* PA14. Cells were
681 grown at 37°C in sterile M9 minimal medium supplemented with 0.2% glucose and
682 0.1% casamino acids. All antibiotics were prepared according to manufacturer's
683 instructions (Table 1). All experiments were carried out in 96-well plates shaken and
684 incubated at 37°C for 12h and treatments being randomized across each plate. After
685 12h of growth, optical density (OD) measurements were taken in BioTek plate readers.
686 Randomization schemes of plates for each experiment were different from each other.
687

688 **Selection of highly resistant mutants**

689 We challenged 20 isogenic populations of PA14 against increasing concentrations of
690 each of the studied antibiotics (160 populations in total; Fig. 1A). As a control for the
691 adaptation to growth in medium without antibiotics we also included 20 populations
692 growing in M9 only, resulting in a total of 180 populations. Experiments were initiated
693 with half the antibiotic concentration that causes growth inhibition of at least 90%
694 (IC90) for each drug in a final volume of 100µl per well. Every 12h 50% volume was
695 transferred into a freshly prepared plate. Every fourth transfer (4 generations), OD was
696 measured and the antibiotic concentration was increased 1.5 times. Experiments were
697 concluded when reaching ~40 times the IC90 (48 transfers) or when 10 or less of the
698 20 populations had reached extinction. Whenever the antibiotic concentration was
699 increased, the preceding plates were frozen at -80°C in 1:4 (v/v) of 86% glycerol.
700

701 **Fitness measurements**

702 All evolved populations were grown overnight (ON) at 37˚C and 180rpm in M9 media
703 with the corresponding antibiotic they were adapted to. Cultures were then centrifuged,
704 washed with fresh M9 media and diluted in 100µl of M9 without any drug to a starting
705 OD of 0.08 (~10^5 CFU/mL). For each population, 4 technical replicates were
706 considered, and then incubated inside a plate reader at 37˚C for 24h. Within the plate
707 reader, OD measurements were taken at regular intervals of 15 min, for a total of 96
708 measurements per population and replicate. The collected growth data was then
709 analyzed using the R package 'grofit' to obtain three growth parameters: growth rate,
710 length of lag time, and maximum yield. We standardized the fitness of each population
711 relative to the mean of the evolved controls (adapted to M9 media only).

712

713 **Collateral sensitivity and resistance assays**

714 We measured collateral effects for the experimentally evolved populations with high
715 levels of resistance. We tested these populations and also the ancestral PA14 against 10
716 different concentrations of a given antibiotic in randomized order (each concentration
717 was replicated 3 times; total of 21120 concentration and population combinations). To
718 quantify the change in resistance or sensitivity we first considered growth relative to
719 the no-drug environment in order to account for differences in fitness defects among
720 populations originated after adaptation. We then calculated the area under the curve
721 using a spline approximation of the ancestral PA14 and subtracted the one obtained for
722 each population. Positive values reflect a higher cumulative growth at increasing
723 concentrations in the population compared to the ancestor, thus indicating cross-
724 resistance; conversely, negative values represent collateral sensitivity. To derive
725 significant sensitivity or resistance, we performed a Wilcox rank test for all populations
726 adapted to a given environment when challenged against a given antibiotic, comparing
727 significant differences from 0. *P*-values were adjusted for multiple testing using the
728 false discovery rate, FDR (supplementary fig. S5). We subsequently asked how many
729 cases of direct or indirect adaptation had occurred for each antibiotic. Direct adaptation
730 considers how often adaptation to a given drug leads to resistance (or sensitivity)
731 against other drugs, whilst indirect adaptation reflects how often adaptation to other
732 drugs lead to resistance (or sensitivity) against a particular antibiotic. For example, we
733 asked how many cases of resistance against all other drugs were observed upon
734 adaptation to CIP (direct adaptation); as well as how many cases of resistance against
735 CIP were found upon adaptation to any other drug (indirect adaptation).

736

737 **Chemical similarity**

738 We inferred chemical relatedness as previously described by using the Jaccard's
739 similarity index contrasting the chemical fingerprints of all antimicrobial compounds
740 used here (Lázár et al. 2014). We then correlated these pairwise comparisons to the
741 frequency of cross-resistance calculated by:

742

743 $$FCR = \left(R_{A\to B} + R_{B\to A}\right) / L_{AB},$$

744

745 where $R_{A \to B}$ is the number of populations resistant to drug A with cross-resistance to
746 drug B, $R_{B \to A}$ is the number of populations resistant to drug B with cross-resistance to
747 A, and L_{AB} is the total number of populations adapted to A and B.
748
749
750 **DNA extraction**
751 To identify the genetic changes leading to high-level resistance evolution, we
752 sequenced full genomes for whole populations of the ancestral *P. aeruginosa* PA14, 10
753 evolved controls and 71 populations adapted to different drugs. Frozen material from
754 all populations was thawed, and 10µl of each were transferred into 15 ml of M9 minimal
755 medium with the corresponding antibiotic. All populations were shaken and incubated
756 at 37˚C ON. DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen,
757 Hilden, Germany) following manufacturer's recommendations for Gram-negative
758 bacteria. Eighty-one populations in total were sequenced at the Institute for Clinical
759 Microbiology, Kiel University Hospital, using the Illumina HiSeq paired-end
760 technology (Bentley et al. 2008) with an insert size of 150bp and 300x coverage.
761
762 **Genomic analysis**
763 We employed an established pipeline encoded in serial bash and Perl scripts used
764 previously for the genomic analysis of *P. aeruginosa* PA14 (Jansen et al. 2015). Briefly,
765 reads with unreliable quality were removed using Skewer (Jiang et al. 2014). Samples
766 were then mapped to the published *P. aeruginosa*_UCBPP_PA14_uid57977 reference
767 genome available at
768 (ftp://ftp.ncbi.nih.gov/genomes/Bacteria/*Pseudomonas_aeruginosa*_UCBPP_PA14_u
769 id57977). Mapping was performed using bwa and samtools (Li et al. 2009; Li and
770 Durbin 2010) and then visually inspected for low-quality areas using IGV (Integrated
771 genome viewer, Broad Institute; www.broadinstitute.org/software/igv/).
772
773 Duplicated regions were removed for single nucleotide polymorphisms and structural
774 variant calling (SNPs and SV) using MarkDuplicates in Picardtools
775 (http://picard.source-forge.net). To call SNPs and small indels above a threshold
776 frequency of 0.1 and base quality above 20 we employed both frequentist and heuristic
777 methods using respectively SNVer and VarScan (Wei et al. 2011; Koboldt et al. 2012).

To identify larger indels and other SV, we used Pindel and CNVnator (Ye et al. 2009; Abyzov et al. 2011). The resulting output files were filtered for duplicates, ancestral variants, and variants found in the evolved controls. We used a combination of sources to identify and annotate the variants using snpEff (Cingolani et al. 2012), DAVID, the *Pseudomonas* database (available online at: http://pseudomonas.com) and information from published work. Further count statistics, analysis and visualizations were done in the R platform (R Core Team).

Mutational diversity was calculated as in (Chevereau et al. 2015). Briefly, we calculated the entropy $H = -\sum [p_j(\log_2 p_j + (1 - p_j \log_2(1 - p_j)]$, where p_j is the probability that a given locus j is mutated in a random population. H then measures the diversity of mutated loci in the populations adapted to a given drug. Standard error was obtained from jackknife resampling in the R platform.

In order to link the observed collateral effects to the underlying genetic changes we performed a hierarchical clustering analysis. For this, we focused on four treatments, which repeatedly produced contrasting patterns of collateral effects. These included populations adapted to either GEN or STR (the two aminoglycosides), which produced variation in their collateral profiles towards PIT and CAR. We also considered the reverse two cases, for which replicated populations that had adapted to PIT and CAR showed contrasting patterns of collateral effects towards GEN and STR. For these four cases, we first obtained the Euclidean similarity of the sensitivities of evolved populations against the considered drugs. Then we used hierarchical clustering based on Ward's minimum variance method, including the Ward's criterion, which aims at finding compact, spherical clusters, and combined it with bootstrapping to asses cluster stability (Murtagh and Legendre 2014). The same process was then used to infer clusters based on the genomic profiles of the same populations, including only genes with mutations within their coding regions. For each antibiotic we then built dendograms for the clustering results and assessed to what extent given genomic clusters coincided with clusters having collateral resistance or sensitivity phenotypes.

The obtained genome sequences are available from NCBI SRA database under the BioProject number: PRJNA355367.

811

812 ***P. aeruginosa* PA14 genome editing**

813 Deletion of *nalC* and single nucleotide mutations in *pmrB* and *mexZ* were performed in
814 *P. aeruginosa* PA14 based on a two-step recombination method previously described
815 (Trebosc et al. 2016). DNA fragments corresponding to 700-bp up- and downstream of
816 the *nalC* region to be deleted (position 1,391,565-1,390,977 on PA14 genome,
817 GenBank CP000438.1) were amplified by PCR using primers oVT464/oVT465 and
818 oVT466/oVT467, respectively. The resulting DNA fragments were introduced into
819 pVT77 previously digested with EcoRI/XbaI using NEBuilder HiFi DNA assembly
820 (New England Biolabs). For the allelic replacement of wildtype *pmrB* and *mexZ*, 1.4-
821 kb DNA fragments were amplified by PCR using primers oVT468/469, oVT470/471
822 and oVT472/473 on the evolved populations GEN-3, GEN-10, and STR-2, which
823 respectively contained the PmrB_V136E, PmrB_P254L and MexZ_Q95stop
824 mutations. The resulting DNA fragments were cloned into pVT77, digested with
825 EcoRI/XbaI, using NEBuilder HiFi DNA assembly.

826

827 The obtained plasmids were transformed into *E. coli* conjugative strains MFD*pir* or
828 S17-1 and transferred into *P. aeruginosa* PA14 as described previously (Trebosc et al.
829 2016). After conjugation, genomic plasmid integration was tested on LB agar plates
830 containing 100 µg/ml sodium tellurite – when *E. coli* S17-1 was used additional 30
831 µg/ml Chloramphenicol were added to selection plates to eradicate the *E. coli* cells.
832 Plasmid integration into the PA14 genome was confirmed by PCR with primers oVT8
833 and oVT474, oVT476, oVT478 and oVT480 for *nalC* deletion, PmrB V136E, PmrB
834 P254L and MexZ Q95stop mutations, respectively. PA14 clones were transferred to
835 LB agar plates containing 1 mM isopropyl-β-D-1-thiogalactopyranoside and 200 µg/ml
836 3'-azido-3'-deoxythymidine to select for plasmid removal from the genome. Clones
837 were screened by PCR using primers oVT474/oVT475, oVT476/oVT477,
838 oVT478/oVT479 and oVT480/oVT481 for *nalC* deletion, PmrB V136E, PmrB P254L
839 and MexZ Q95stop mutations, respectively. The genomic deletion and mutations were
840 finally confirmed by DNA sequencing (Microsynth AG, Balgach, Switzerland).

841

842 **Table 2. Oligonucleotides used**

Oligo name	Sequence (5'-3')
oVT8	GTTTTCCCAGTCACGACGC

oVT464	AGAATTGAGGCCTCTCGAGGAATTCTTAGAGGTCCCAGGCATTG
oVT465	TGAGGAACAGGGTTTGCTGAGAGCGTTTC
oVT466	TCAGCAAACCCTGTTCCTCAAGGCCCTC
oVT467	CCGCAAGCTTCCTGCAGGCTCTAGACTGATGGAAACCTTTGCC
oVT468	AGAATTGAGGCCTCTCGAGGAATTCTCGATCTCGACGAACTGC
oVT469	CCGCAAGCTTCCTGCAGGCTCTAGACAACGACAGCTCGATGTC
oVT470	AGAATTGAGGCCTCTCGAGGAATTCCTCTCGCTGAAGCAGGTG
oVT471	CCGCAAGCTTCCTGCAGGCTCTAGAATCATCTTCGGCGTCAGTC
oVT472	AGAATTGAGGCCTCTCGAGGAATTCTCCTGGCCTTCCTCGTAC
oVT473	CCGCAAGCTTCCTGCAGGCTCTAGAAGGTAGGCGGAGAAAACG
oVT474	TCTGCGCGGATTCTGATAGC
oVT475	TCCCTGGAAATGCAGTGAGC
oVT476	GACGACTACCTGACCAAGCC
oVT477	CCTTCAGCCACAGGTCGATG
oVT478	CGAGGTCCATGTCCATCACC
oVT479	TCGTTCTCGTTGTAGTGGCG
oVT480	CCTTGATCAGGTCGGCGTAG
oVT481	AAGCTACCGTGACAGAACCC

843
844
845 **Acknowledgements**
846
847 We thank Anette Friedrichs, Lutz Becks, and the Schulenburg group for valuable advice
848 and Melanie Vollstedt for technical support during genome sequencing. We are grateful
849 for financial support from the German Science Foundation (DFG grant SCHU 1415/12-
850 1) and the International Max-Planck Research School for Evolutionary Biology at the
851 University of Kiel. We acknowledge infrastructural support by the DFG excellence
852 cluster Inflammation at Interfaces.
853

854 **References**

855 Abyzov A, Urban AE, Snyder M, Gerstein M. 2011. CNVnator: An approach to
856 discover, genotype and characterize typical and atypical CNVs from family
857 and population genome sequencing. Genome Res.:gr.114876.110.

858 Aires JR, Köhler T, Nikaido H, Plésiat P. 1999. Involvement of an Active Efflux
859 System in the Natural Resistance of Pseudomonas aeruginosa to
860 Aminoglycosides. Antimicrob. Agents Chemother. 43:2624–2628.

861 Amin NE, Giske CG, Jalal S, Keijser B, Kronvall G, Wretlind B. 2005. Carbapenem
862 resistance mechanisms in Pseudomonas aeruginosa: alterations of porin
863 OprD and efflux proteins do not fully explain resistance patterns observed
864 in clinical isolates. APMIS 113:187–196.

865 Andersson DI, Hughes D. 2010. Antibiotic resistance and its cost: is it possible to
866 reverse resistance? Nat. Rev. Microbiol. 8:260–271.

867 Arruda EAG MD, Marinho IS MD, Boulos M MD, Sinto SI BSc, F. HHC BSc, Mendes
868 CM MD, Oplustil CP BSc, Sader H MD, Levy CE MD, Levin AS MD. 1999.
869 Nosocomial Infections Caused by Multiresistant Pseudomonas
870 aeruginosa •. Infect. Control Hosp. Epidemiol. 20:620–623.

871 Balasubramanian D, Schneper L, Kumari H, Mathee K. 2013. A dynamic and
872 intricate regulatory network determines Pseudomonas aeruginosa
873 virulence. Nucleic Acids Res. 41:1–20.

874 Barrick JE, Yu DS, Yoon SH, Jeong H, Oh TK, Schneider D, Lenski RE, Kim JF. 2009.
875 Genome evolution and adaptation in a long-term experiment with
876 Escherichia coli. Nature 461:1243–1247.

877 Baym M, Lieberman TD, Kelsic ED, Chait R, Gross R, Yelin I, Kishony R. 2016.
878 Spatiotemporal microbial evolution on antibiotic landscapes. Science
879 353:1147–1151.

880 Baym M, Stone LK, Kishony R. 2016. Multidrug evolutionary strategies to reverse
881 antibiotic resistance. Science 351:aad3292.

882 Bentley DR, Balasubramanian S, Swerdlow HP, Smith GP, Milton J, Brown CG, Hall
883 KP, Evers DJ, Barnes CL, Bignell HR, et al. 2008. Accurate whole human
884 genome sequencing using reversible terminator chemistry. Nature
885 456:53–59.

886 Breidenstein EBM, de la Fuente-Núñez C, Hancock REW. 2011. Pseudomonas
887 aeruginosa: all roads lead to resistance. Trends Microbiol. 19:419–426.

888 Burrows LL. 2012. Pseudomonas aeruginosa Twitching Motility: Type IV Pili in
889 Action. Annu. Rev. Microbiol. 66:493–520.

890 Cabot G, Zamorano L, Moyà B, Juan C, Navas A, Blázquez J, Oliver A. 2016.
891 Evolution of Pseudomonas aeruginosa Antimicrobial Resistance and

892 Fitness under Low and High Mutation Rates. Antimicrob. Agents
893 Chemother. 60:1767–1778.

894 Cao L, Srikumar R, Poole K. 2004. MexAB-OprM hyperexpression in NalC-type
895 multidrug-resistant Pseudomonas aeruginosa: identification and
896 characterization of the nalC gene encoding a repressor of PA3720-
897 PA3719. Mol. Microbiol. 53:1423–1436.

898 Carmeli Y, Troillet N, Eliopoulos GM, Samore MH. 1999. Emergence of Antibiotic-
899 Resistant Pseudomonas aeruginosa: Comparison of Risks Associated with
900 Different Antipseudomonal Agents. Antimicrob. Agents Chemother.
901 43:1379–1382.

902 Chait R, Craney A, Kishony R. 2007. Antibiotic interactions that select against
903 resistance. Nature 446:668–671.

904 Chevereau G, Dravecká M, Batur T, Guvenek A, Ayhan DH, Toprak E, Bollenbach
905 T. 2015. Quantifying the Determinants of Evolutionary Dynamics Leading
906 to Drug Resistance. PLoS Biol 13:e1002299.

907 Cingolani P, Platts A, Wang LL, Coon M, Nguyen T, Wang L, Land SJ, Lu X, Ruden
908 DM. 2012. A program for annotating and predicting the effects of single
909 nucleotide polymorphisms, SnpEff: SNPs in the genome of Drosophila
910 melanogaster strain w1118; iso-2; iso-3. Fly (Austin) 6:80–92.

911 Ciofu O, Mandsberg LF, Bjarnsholt T, Wassermann T, Høiby N. 2010. Genetic
912 adaptation of Pseudomonas aeruginosa during chronic lung infection of
913 patients with cystic fibrosis: strong and weak mutators with
914 heterogeneous genetic backgrounds emerge in mucA and/or lasR
915 mutants. Microbiology 156:1108–1119.

916 Daigle DM, Cao L, Fraud S, Wilke MS, Pacey A, Klinoski R, Strynadka NC, Dean CR,
917 Poole K. 2007. Protein Modulator of Multidrug Efflux Gene Expression in
918 Pseudomonas aeruginosa. J. Bacteriol. 189:5441–5451.

919 Davies J, Davies D. 2010. Origins and Evolution of Antibiotic Resistance.
920 Microbiol. Mol. Biol. Rev. 74:417–433.

921 Doyle TB, Hawkins AC, McCarter LL. 2004. The Complex Flagellar Torque
922 Generator of Pseudomonas aeruginosa. J. Bacteriol. 186:6341–6350.

923 Drenkard E, Ausubel FM. 2002. Pseudomonas biofilm formation and antibiotic
924 resistance are linked to phenotypic variation. Nature 416:740–743.

925 Evgrafov MR de, Gumpert H, Munck C, Thomsen TT, Sommer MOA. 2015.
926 Collateral Resistance and Sensitivity Modulate Evolution of High-Level
927 Resistance to Drug Combination Treatment in Staphylococcus aureus.
928 Mol. Biol. Evol. 32:1175–1185.

929 Folkesson A, Jelsbak L, Yang L, Johansen HK, Ciofu O, Høiby N, Molin S. 2012.
930 Adaptation of Pseudomonas aeruginosa to the cystic fibrosis airway: an
931 evolutionary perspective. Nat. Rev. Microbiol. 10:841–851.

932 Fuentes-Hernandez A, Plucain J, Gori F, Pena-Miller R, Reding C, Jansen G,
933 Schulenburg H, Gudelj I, Beardmore R. 2015. Using a Sequential Regimen
934 to Eliminate Bacteria at Sublethal Antibiotic Dosages. PLoS Biol
935 13:e1002104.

936 Furuya EY, Lowy FD. 2006. Antimicrobial-resistant bacteria in the community
937 setting. Nat. Rev. Microbiol. 4:36–45.

938 Gotoh N, Tsujimoto H, Tsuda M, Okamoto K, Nomura A, Wada T, Nakahashi M,
939 Nishino T. 1998. Characterization of the MexC-MexD-OprJ Multidrug
940 Efflux System in ΔmexA-mexB-oprM Mutants of Pseudomonas
941 aeruginosa. Antimicrob. Agents Chemother. 42:1938–1943.

942 Govan JR, Deretic V. 1996. Microbial pathogenesis in cystic fibrosis: mucoid
943 Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol. Rev.
944 60:539–574.

945 Hancock REW, Speert DP. 2000. Antibiotic resistance in Pseudomonas
946 aeruginosa: mechanisms and impact on treatment. Drug Resist. Updat.
947 3:247–255.

948 Hauser AR. 2009. The type III secretion system of Pseudomonas aeruginosa:
949 infection by injection. Nat. Rev. Microbiol. 7:654–665.

950 Hegreness M, Shoresh N, Damian D, Hartl D, Kishony R. 2008. Accelerated
951 evolution of resistance in multidrug environments. Proc. Natl. Acad. Sci.
952 105:13977–13981.

953 Hoch JA. 2000. Two-component and phosphorelay signal transduction. Curr.
954 Opin. Microbiol. 3:165–170.

955 Imamovic L, Sommer MOA. 2013. Use of Collateral Sensitivity Networks to
956 Design Drug Cycling Protocols That Avoid Resistance Development. Sci.
957 Transl. Med. 5:204ra132-204ra132.

958 Jansen G, Barbosa C, Schulenburg H. 2013. Experimental evolution as an efficient
959 tool to dissect adaptive paths to antibiotic resistance. Drug Resist. Updat.
960 16:96–107.

961 Jansen G, Crummenerl LL, Gilbert F, Mohr T, Pfefferkorn R, Thänert R, Rosenstiel
962 P, Schulenburg H. 2015. Evolutionary Transition from Pathogenicity to
963 Commensalism: Global Regulator Mutations Mediate Fitness Gains
964 through Virulence Attenuation. Mol. Biol. Evol. 32:2883–2896.

965 Jeannot K, Elsen S, Köhler T, Attree I, van Delden C, Plésiat P. 2008. Resistance
966 and Virulence of Pseudomonas aeruginosa Clinical Strains Overproducing

967 the MexCD-OprJ Efflux Pump. Antimicrob. Agents Chemother. 52:2455–
968 2462.

969 Jiang H, Lei R, Ding S-W, Zhu S. 2014. Skewer: a fast and accurate adapter
970 trimmer for next-generation sequencing paired-end reads. BMC
971 Bioinformatics 15:182.

972 Jimenez PN, Koch G, Thompson JA, Xavier KB, Cool RH, Quax WJ. 2012. The
973 Multiple Signaling Systems Regulating Virulence in Pseudomonas
974 aeruginosa. Microbiol. Mol. Biol. Rev. 76:46–65.

975 Kang C-I, Kim S-H, Kim H-B, Park S-W, Choe Y-J, Oh M, Kim E-C, Choe K-W. 2003.
976 Pseudomonas aeruginosa Bacteremia: Risk Factors for Mortality and
977 Influence of Delayed Receipt of Effective Antimicrobial Therapy on
978 Clinical Outcome. Clin. Infect. Dis. 37:745–751.

979 Kim S, Lieberman TD, Kishony R. 2014. Alternating antibiotic treatments
980 constrain evolutionary paths to multidrug resistance. Proc. Natl. Acad. Sci.
981 111:14494–14499.

982 Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, Miller CA, Mardis
983 ER, Ding L, Wilson RK. 2012. VarScan 2: Somatic mutation and copy
984 number alteration discovery in cancer by exome sequencing. Genome Res.
985 [Internet]. Available from:
986 http://genome.cshlp.org/content/early/2012/02/02/gr.129684.111

987 Korotkov KV, Sandkvist M, Hol WGJ. 2012. The type II secretion system:
988 biogenesis, molecular architecture and mechanism. Nat. Rev. Microbiol.
989 10:336–351.

990 Kugelberg E, Löfmark S, Wretlind B, Andersson DI. 2005. Reduction of the fitness
991 burden of quinolone resistance in Pseudomonas aeruginosa. J.
992 Antimicrob. Chemother. 55:22–30.

993 Kussell E. 2013. Evolution in Microbes. Annu. Rev. Biophys. 42:493–514.

994 Larson E. 2007. Community Factors in the Development of Antibiotic Resistance.
995 Annu. Rev. Public Health 28:435–447.

996 Lázár V, Nagy I, Spohn R, Csörgő B, Györkei Á, Nyerges Á, Horváth B, Vörös A,
997 Busa-Fekete R, Hrtyan M, et al. 2014. Genome-wide analysis captures the
998 determinants of the antibiotic cross-resistance interaction network. Nat.
999 Commun. 5:4352.

1000 Lázár V, Singh GP, Spohn R, Nagy I, Horváth B, Hrtyan M, Busa-Fekete R, Bogos B,
1001 Méhi O, Csörgő B, et al. 2013. Bacterial evolution of antibiotic
1002 hypersensitivity. Mol. Syst. Biol. [Internet] 9. Available from:
1003 http://msb.embopress.org/content/9/1/700

1004 Lenski RE, Rose MR, Simpson SC, Tadler SC. 1991. Long-Term Experimental
1005 Evolution in Escherichia coli. I. Adaptation and Divergence During 2,000
1006 Generations. Am. Nat. 138:1315–1341.

1007 Levin-Reisman I, Ronin I, Gefen O, Braniss I, Shoresh N, Balaban NQ. 2017.
1008 Antibiotic tolerance facilitates the evolution of resistance.
1009 Science:eaaj2191.

1010 Li H, Durbin R. 2010. Fast and accurate long-read alignment with Burrows–
1011 Wheeler transform. Bioinformatics 26:589–595.

1012 Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G,
1013 Durbin R, Subgroup 1000 Genome Project Data Processing. 2009. The
1014 Sequence Alignment/Map format and SAMtools. Bioinformatics 25:2078–
1015 2079.

1016 Li X-Z, Zhang L, Poole K. 2000. Interplay between the MexA-MexB-OprM
1017 multidrug efflux system and the outer membrane barrier in the multiple
1018 antibiotic resistance of Pseudomonas aeruginosa. J. Antimicrob.
1019 Chemother. 45:433–436.

1020 Livermore DM. 2002. Multiple Mechanisms of Antimicrobial Resistance in
1021 Pseudomonas aeruginosa: Our Worst Nightmare? Clin. Infect. Dis.
1022 34:634–640.

1023 Loper JE, Hassan KA, Mavrodi DV, Ii EWD, Lim CK, Shaffer BT, Elbourne LDH,
1024 Stockwell VO, Hartney SL, Breakwell K, et al. 2012. Comparative Genomics
1025 of Plant-Associated Pseudomonas spp.: Insights into Diversity and
1026 Inheritance of Traits Involved in Multitrophic Interactions. PLOS Genet
1027 8:e1002784.

1028 Ma L, Conover M, Lu H, Parsek MR, Bayles K, Wozniak DJ. 2009. Assembly and
1029 Development of the Pseudomonas aeruginosa Biofilm Matrix. PLoS
1030 Pathog 5:e1000354.

1031 Macfarlane ELA, Kwasnicka A, Hancock REW. 2000. Role of Pseudomonas
1032 aeruginosa PhoP-PhoQ in resistance to antimicrobial cationic peptides
1033 and aminoglycosides. Microbiology 146:2543–2554.

1034 Maseda H, Yoneyama H, Nakae T. 2000. Assignment of the Substrate-Selective
1035 Subunits of the MexEF-OprN Multidrug Efflux Pump of Pseudomonas
1036 aeruginosa. Antimicrob. Agents Chemother. 44:658–664.

1037 Masuda N, Sakagawa E, Ohya S, Gotoh N, Tsujimoto H, Nishino T. 2000. Substrate
1038 Specificities of MexAB-OprM, MexCD-OprJ, and MexXY-OprM Efflux
1039 Pumps in Pseudomonas aeruginosa. Antimicrob. Agents Chemother.
1040 44:3322–3327.

1041 McPhee JB, Lewenza S, Hancock REW. 2003. Cationic antimicrobial peptides
1042 activate a two-component regulatory system, PmrA-PmrB, that regulates

1043 resistance to polymyxin B and cationic antimicrobial peptides in
1044 Pseudomonas aeruginosa. Mol. Microbiol. 50:205–217.

1045 Melnyk AH, Wong A, Kassen R. 2015. The fitness costs of antibiotic resistance
1046 mutations. Evol. Appl. 8:273–283.

1047 Michel J-B, Yeh PJ, Chait R, Moellering RC, Kishony R. 2008. Drug interactions
1048 modulate the potential for evolution of resistance. Proc. Natl. Acad. Sci.
1049 105:14918–14923.

1050 Miller C, Thomsen LE, Gaggero C, Mosseri R, Ingmer H, Cohen SN. 2004. SOS
1051 Response Induction by ß-Lactams and Bacterial Defense Against
1052 Antibiotic Lethality. Science 305:1629–1631.

1053 Montanari S, Oliver A, Salerno P, Mena A, Bertoni G, Tümmler B, Cariani L, Conese
1054 M, Döring G, Bragonzi A. 2007. Biological cost of hypermutation in
1055 Pseudomonas aeruginosa strains from patients with cystic fibrosis.
1056 Microbiology 153:1445–1454.

1057 Munck C, Gumpert HK, Wallin AIN, Wang HH, Sommer MOA. 2014. Prediction of
1058 resistance development against drug combinations by collateral
1059 responses to component drugs. Sci. Transl. Med. 6:262ra156-262ra156.

1060 Murtagh F, Legendre P. 2014. Ward's Hierarchical Agglomerative Clustering
1061 Method: Which Algorithms Implement Ward's Criterion? J. Classif.
1062 31:274–295.

1063 Nikel PI, Martínez-García E, de Lorenzo V. 2014. Biotechnological domestication
1064 of pseudomonads using synthetic biology. Nat. Rev. Microbiol. 12:368–
1065 379.

1066 Oliver A, Baquero F, Blázquez J. 2002. The mismatch repair system (mutS, mutL
1067 and uvrD genes) in Pseudomonas aeruginosa: molecular characterization
1068 of naturally occurring mutants. Mol. Microbiol. 43:1641–1650.

1069 Overhage J, Bains M, Brazas MD, Hancock REW. 2008. Swarming of Pseudomonas
1070 aeruginosa Is a Complex Adaptation Leading to Increased Production of
1071 Virulence Factors and Antibiotic Resistance. J. Bacteriol. 190:2671–2679.

1072 Oz T, Guvenek A, Yildiz S, Karaboga E, Tamer YT, Mumcuyan N, Ozan VB, Senturk
1073 GH, Cokol M, Yeh P, et al. 2014. Strength of Selection Pressure Is an
1074 Important Parameter Contributing to the Complexity of Antibiotic
1075 Resistance Evolution. Mol. Biol. Evol. 31:2387–2401.

1076 Pál C, Papp B, Lázár V. 2015. Collateral sensitivity of antibiotic-resistant
1077 microbes. Trends Microbiol. 23:401–407.

1078 Pena-Miller R, Laehnemann D, Jansen G, Fuentes-Hernandez A, Rosenstiel P,
1079 Schulenburg H, Beardmore R. 2013. When the Most Potent Combination
1080 of Antibiotics Selects for the Greatest Bacterial Load: The Smile-Frown
1081 Transition. PLoS Biol 11:e1001540.

1082 Piddock LJV. 2006. Multidrug-resistance efflux pumps ? not just for resistance.
1083 Nat. Rev. Microbiol. 4:629–636.

1084 Poole K. 2001. Multidrug efflux pumps and antimicrobial resistance in
1085 Pseudomonas aeruginosa and related organisms. J Mol Microb. Biotechnol
1086 3:255–264.

1087 R Core Team. R: A Language and Environment for Statistical Computing. Vienna,
1088 Austria: R Foundation for Statistical Computing Available from:
1089 https://www.R-project.org/

1090 Ramos JL, Martínez-Bueno M, Molina-Henares AJ, Terán W, Watanabe K, Zhang X,
1091 Gallegos MT, Brennan R, Tobes R. 2005. The TetR Family of
1092 Transcriptional Repressors. Microbiol. Mol. Biol. Rev. 69:326–356.

1093 Robinson VL, Buckler DR, Stock AM. 2000. A tale of two components: a novel
1094 kinase and a regulatory switch. Nat. Struct. Mol. Biol. 7:626–633.

1095 Rodrigue A, Quentin Y, Lazdunski A, Méjean V, Foglino M. 2000. Cell signalling by
1096 oligosaccharides. Two-component systems in Pseudomonas aeruginosa:
1097 why so many? Trends Microbiol. 8:498–504.

1098 Rodríguez-Rojas A, Oliver A, Blázquez J. 2012. Intrinsic and Environmental
1099 Mutagenesis Drive Diversification and Persistence of Pseudomonas
1100 aeruginosa in Chronic Lung Infections. J. Infect. Dis. 205:121–127.

1101 Roemhild R, Barbosa C, Beardmore RE, Jansen G, Schulenburg H. 2015. Temporal
1102 variation in antibiotic environments slows down resistance evolution in
1103 pathogenic Pseudomonas aeruginosa. Evol. Appl.:n/a-n/a.

1104 Szybalski W, Bryson V. 1952. GENETIC STUDIES ON MICROBIAL CROSS
1105 RESISTANCE TO TOXIC AGENTS I. ,. J. Bacteriol. 64:489–499.

1106 Tenaillon O, Barrick JE, Ribeck N, Deatherage DE, Blanchard JL, Dasgupta A, Wu
1107 GC, Wielgoss S, Cruveiller S, Médigue C, et al. 2016. Tempo and mode of
1108 genome evolution in a 50,000-generation experiment. Nature 536:165–
1109 170.

1110 Toprak E, Veres A, Michel J-B, Chait R, Hartl DL, Kishony R. 2012. Evolutionary
1111 paths to antibiotic resistance under dynamically sustained drug selection.
1112 Nat. Genet. 44:101–105.

1113 Trebosc V, Gartenmann S, Royet K, Manfredi P, Tötzl M, Schellhorn B, Pieren M,
1114 Tigges M, Lociuro S, Sennhenn PC, et al. 2016. A Novel Genome-Editing
1115 Platform for Drug-Resistant Acinetobacter baumannii Reveals an AdeR-
1116 Unrelated Tigecycline Resistance Mechanism. Antimicrob. Agents
1117 Chemother. 60:7263–7271.

1118 Tsutsumi Y, Tomita H, Tanimoto K. 2013. Identification of Novel Genes
1119 Responsible for Overexpression of ampC in Pseudomonas aeruginosa
1120 PAO1. Antimicrob. Agents Chemother. 57:5987–5993.

1121 Wei Z, Wang W, Hu P, Lyon GJ, Hakonarson H. 2011. SNVer: a statistical tool for
1122 variant calling in analysis of pooled or individual next-generation
1123 sequencing data. Nucleic Acids Res. 39:e132–e132.

1124 Westbrock-Wadman S, Sherman DR, Hickey MJ, Coulter SN, Zhu YQ, Warrener P,
1125 Nguyen LY, Shawar RM, Folger KR, Stover CK. 1999. Characterization of a
1126 Pseudomonas aeruginosa Efflux Pump Contributing to Aminoglycoside
1127 Impermeability. Antimicrob. Agents Chemother. 43:2975–2983.

1128 Ye K, Schulz MH, Long Q, Apweiler R, Ning Z. 2009. Pindel: a pattern growth
1129 approach to detect break points of large deletions and medium sized
1130 insertions from paired-end short reads. Bioinformatics 25:2865–2871.

1131