Experimental evolution to identify undescribed mechanisms of resistance to a novel cationic peptide antibiotic

Santos-Lopez, A[1,2,†,*,a], Fritz MJ[1,†]., Lombardo J[1]., Burr AHP[1], Heinrich VA[1], Marshall CW[1,2,b], Cooper VS[1,2*].

[1]*Department of Microbiology and Molecular Genetics, and Center for Evolutionary Biology and Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA.*
[2]*Center for Evolutionary Biology and Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania, USA.*
[a] *Current Address: Department of Microbiology, Hospital Universitario Ramon y Cajal, Instituto Ramón y Cajal de Investigación Sanitaria (IRYCIS), Madrid, Spain*
[b] *Current Address: Department of Biological Sciences, Marquette University, Milwaukee, Wisconsin, USA*

† Both authors have contributed equally
*Corresponding authors: Alfonso Santos-Lopez, and Vaughn Cooper

Abstract

Introduction: A key strategy for resolving the antibiotic resistance crisis is the development of new drugs with antimicrobial properties. The engineered cationic antimicrobial peptide WLBU2 (also known as PLG206) is a promising compound that can clear Gram-negative and Gram-positive populations and infections even when bacteria are embedded in biofilms. Despite several attempts, no definitive mechanisms of resistance to WLBU2 had been found. Here, we used experimental evolution followed by whole genome sequencing to detect novel genetic pathways and probable mechanisms of resistance to this peptide.

Methods and Results: We propagated populations of wild-type and mutator *Pseudomonas aeruginosa* in the presence of WLBU2 and performed whole genome sequencing (WGS) of evolved populations. Populations that survived WLBU2 treatment acquired a minimum of two mutations, making the acquisition of resistance more difficult than for most antibiotics, which can be tolerated by mutation of a single target. Major targets included the *orfN* and *pmrB* genes, previously described to confer resistance to other cationic peptides. More surprisingly, mutations that increase aggregation such as the *wsp* pathway were also selected despite the ability of WLBU2 to kill cells growing in a biofilm.

Conclusion: Experimental evolution and WGS identifies genetic targets and actions of new antimicrobial compounds and can predict pathways to resistance to new antibiotics in clinical practice.

Introduction

The ability of a bacterial population to evolve resistance to an antibiotic depends on several factors including the availability of mutations that increase resistance and the strength of selection imposed by the compound. Experimental evolution (EE) coupled with whole genome sequencing (WGS) is a powerful strategy to characterize the genetic mechanisms of resistance. Propagation of a bacterial population in the presence of an antibiotic will eventually select those clones that are capable of surviving the antibiotic exposure, and WGS of these populations or clones will reveal genetic causes of the resistance phenotype (Barbosa et al., 2019; Gullberg et al., 2011; Maltas and Wood, 2019; Santos-Lopez et al., 2019). This method is especially powerful when studying cationic peptides, as multiple mutations may be needed to evolve resistance to them (Mehta et al., 2018; Perron et al., 2006; Spohn et al., 2019).

WLBU2 (also called PLG206) is an engineered amphipathic alpha helix derived from the LL-37 peptide that inserts into the bacterial membrane and leads to cell death. WLBU2 is shown to be highly effective against ESKAPE (***E****nterococcus faecium,* ***S****taphylococcus aureus,* ***K****lebsiella pneumoniae,* ***A****cinetobacter baumannii,* ***P****seudomonas aeruginosa* and ***E****nterobacter spp.*) pathogens *in vitro* and *in vivo* even when the bacterial populations are protected by biofilms (Chen et al., 2018; Deslouches et al., 2015; Di et al., 2020; Kumagai et al., 2019; Lashua et al., 2016; Lin et al., 2018; Mandell et al., 2017, 2020; Melvin et al., 2016; Paranjape et al., 2013; Swedan et al., 2019). Despite challenging several Gram-negative and Gram-positive pathogens with WLBU2 *in vitro* and in animal models (see previous references), only one study has shown that *P. aeruginosa* was able to increase resistance following long exposure to WLBU2 (Deslouches et al., 2015). However, no mechanisms of resistance have been described yet. Identifying the genes and the subsequent mutations that confer resistance to novel antibiotics is crucial as it enables more comprehensive understanding of this new antimicrobial compound's actions and how resistance evolves to them. Further, these discoveries can increase our ability to predict the emergence of antimicrobial resistance (AMR) in clinical scenarios (Brockhurst et al., 2019; MacLean, 2020; MacLean and San Millan, 2019).

Results

Failure to evolve resistance using laboratory strains

To identify mechanisms of resistance to WLBU2, we propagated the laboratory strains of *A. baumannii* ATCC 17978 and *P. aeruginosa* PA14 that had no prior exposure to WLBU2. We followed a protocol successfully used previously with several pathogens and different antibiotics, in which large populations of bacteria are treated with increasing concentrations of antibiotic over 12 days (Hernando-Amado et al., 2019; Santos-Lopez et al., 2019, 2020; Scribner et al., 2020; Trampari et al., 2019). We serially passaged both strains (bottleneck of ~10^7 cfu) in WBLU2 concentrations initially at half of the Minimum Inhibitory Concentration (MIC) and increasing two-fold every three days, up to four times the MIC. We propagated the same number of populations in the absence of WLBU2 to distinguish between adaptation to the environment and WLBU2-related mutations. Knowing that biofilm formation can influence the evolution of resistance (Ahmed et al., 2018; Santos-Lopez et al., 2019; Scribner et al., 2020), we implemented this regimen in both planktonic and biofilm cultures (see http://evolvingstem.org and (Santos-Lopez et al., 2019; Scribner et al., 2020) for details of the biofilm propagation). To test the general capability of the experimental design for selecting populations resistant to cationic peptides, we also propagated populations of *A. baumannii* in each lifestyle with increasing concentrations of polymyxin B. While all planktonic populations and four out of five biofilm populations survived in 4-times the MIC of polymyxin B, mainly through mutations in the *pmrABC* operon and in genes modifying lipid A (Table S1), no populations of *A. baumannii* nor *P. aeruginosa* survived exposure to the MIC of WLBU2. This suggested that there may be a narrow genetic pathway to gain WBLU2 resistance that could require more than one mutation.

To increase the likelihood of developing resistance we analyzed the two factors mainly evolution of antibiotic depends on (Hughes and Andersson, 2017): i) the mutation supply determined by population size and mutation rate and ii) the strength of selection imposed by the antibiotic. If we assume a conservative mutation rate of 0.001 mutations per genome per generation, and a Poisson distribution of the mutations in the genome (Dillon et al., 2017), this experiment only had a cumulative probability of ~0.35 for *A. baumannii* and 0.29 for *P. aeruginosa* that any given nucleotide had been mutated at least once following three days of growth (~36 generations) in subinhibitory concentrations, *i.e.* before being exposed to lethal concentrations (Table S2 for detailed calculations). It is therefore possible that the correct mutation or

100 combination of mutations needed to survive the antibiotic treatment had not occurred before facing
101 inhibitory concentrations. We therefore pursued a focused approach with *P. aeruginosa* to increase
102 the number of sampled mutations to determine how resistance could emerge to WLBU2.
103
104 **Increasing mutation supply by using a hypermutator strain promotes evolution of resistance**
105 One way to increase the mutation supply during evolution experiments is to use an
106 ancestral strain with a higher mutation rate (Gifford et al., 2019; MacLean, 2020; de Visser et al.,
107 1999). These mutator genotypes also commonly evolve during chronic infections of *P. aeruginosa*
108 and are therefore relevant to AMR evolution (Mena et al., 2008; Oliver et al., 2000). We selected
109 a strain of *P. aeruginosa* PA14 with a defect in mismatch repair (*mutS* T112P), producing a
110 mutation rate ~100 times higher than the ancestor (Flynn et al., 2016). Under the same
111 experimental conditions described above, each nucleotide experiences >40 mutations over the first
112 three days of growth under subinhibitory conditions (Table S2). Five mutator populations were
113 propagated in each lifestyle combination following the previously described protocol. Two
114 planktonic and three biofilm populations survived this treatment, resulting in 3 - 7.5-fold increases
115 in resistance level relative to the ancestor (Figure 1A).
116 We sequenced genomes from the five surviving populations to a depth of 148.01 ± 42.80
117 and detected a total of 98 mutations at frequencies > 0.1, including 31 fixed mutations (Table S3).
118 As the ancestor was a mutator strain, it can be difficult to infer what mutations were the drivers of
119 the resistance phenotype and which were hitchhikers (Shaver et al., 2002). We focused on
120 instances of gene-level parallel (repeated) evolution as strong candidates, because mutations in the
121 same gene found in independently derived lineages provide strong evidence of selection on this
122 trait. Further, the large population size and high mutation supply empowers selection to enrich the
123 most beneficial genotypes (Bailey et al., 2015; Ibacache-Quiroga et al., 2018; Lieberman et al.,
124 2011; Papkou et al., 2020; Scribner et al., 2020; Toprak et al., 2011; Vogwill et al., 2014).

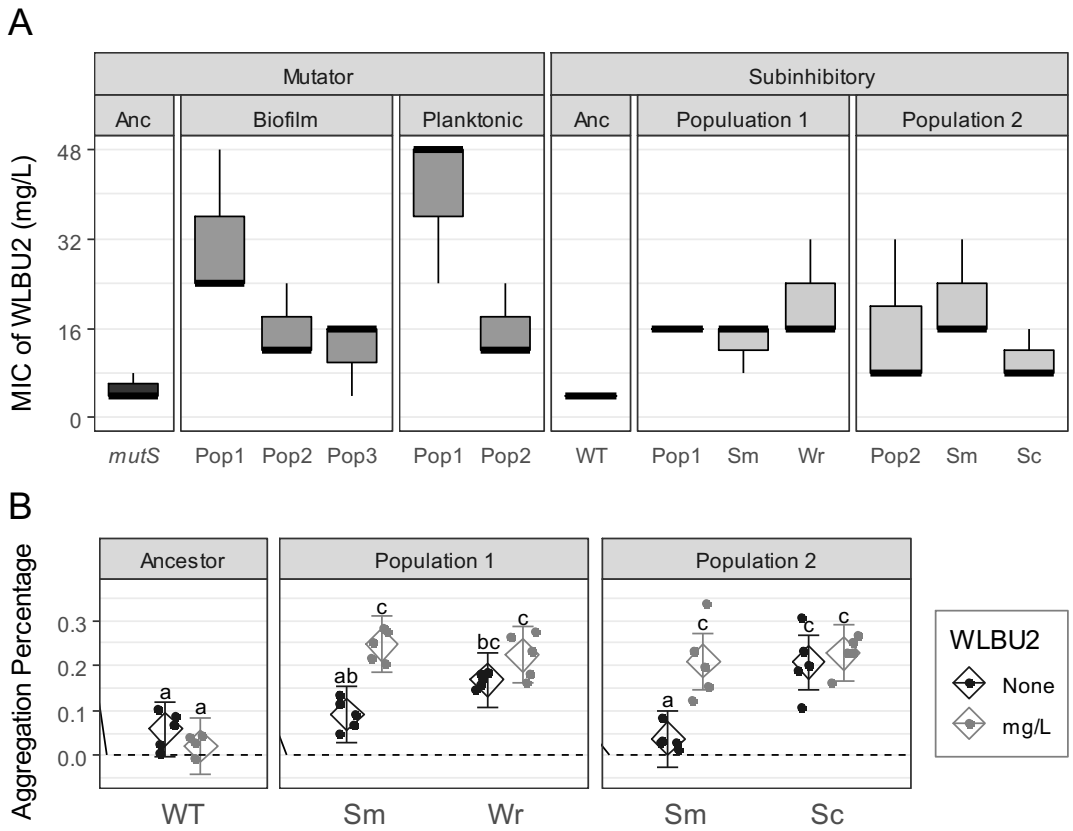

Figure 1. A. Resistance levels of populations evolved in the presence of WLBU2, as minimum inhibitory concentration (MIC) (mg/L). Boxplots show the median and quartiles for 3 replicates. Populations evolved from the *mutS* mutator genotype are in dark grey and resistant clones isolated from the WT ancestor are in light grey. All populations significantly exceed WT resistance ($p < 0.05$, one-way ANOVA following Benjamini et al.) B. Aggregation of PA14 and resistant clones. The resistant Wrinkly (Wr) and Small (Sc) clones (associated with *wsp* mutations) aggregated more than WT regardless of WLBU2 treatement compared, while the Smooth (Sm) clones aggregated more in the presence of WBLU2 only. Aggregation was measured by letting populations of each clone settle for 24 hours at 4ºC, n=5. Means sharing a letter are not significantly different (Sidak-adjusted least-square means for multiple comparisons, $p < 0.05$). Bars show 95% confidence intervals of least-square means.

Only three genes, *pmrB*, *wspF*, and *morA,* were mutated in more than one population exposed to WLBU2 and not in the populations evolving in the absence of antibiotics, indicating roles for these mutations in evading the antibiotic treatment (Table S3). The two-component regulator *pmrB* governs several modifications of lipopolysaccharides (LPS) and has previously been demonstrated to confer resistance to other cationic peptides (Fernández et al., 2013; Moskowitz et al., 2012). Four of the five WLBU2-resistant populations also show cross-resistance to polymyxin B (Figure S1). The *wspF* gene encodes a methylesterase that regulates

activity of the surface-sensing Wsp cluster that in turn activates the diguanylate cyclase WspR and biofilm production (Hickman et al., 2005; Huangyutitham et al., 2013). The *morA* gene encodes multiple sensor domains that control diguanylate cyclase and phosphodiesterase domains acting on the second messenger cyclic di-GMP. This molecule promotes biofilm production at high levels and motility at low levels (Ha and O'Toole, 2015; Hickman et al., 2005). These biofilm-associated mutations strongly indicate that production of aggregates or biofilm plays a role in resisting WLBU2.

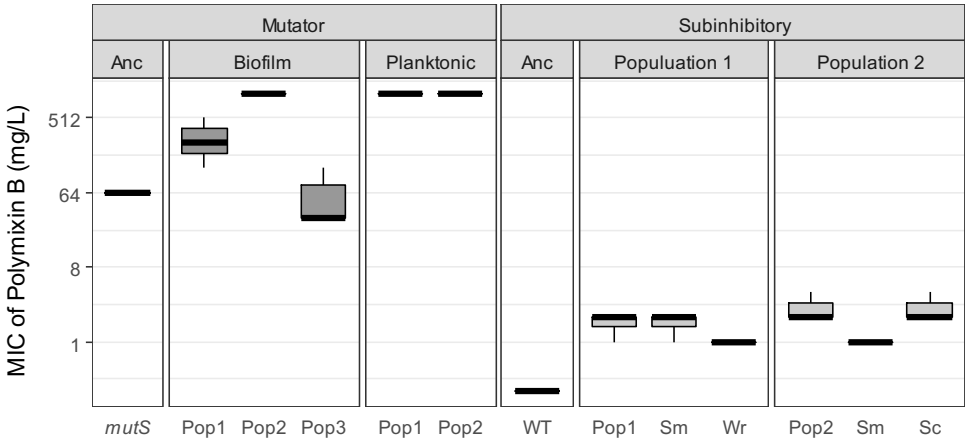

Figure S1. Resistance levels of populations evolved in the presence of polymyxin B, as minimum inhibitory concentration (MIC) (mg/L). Boxplots show the median and quartiles of 3 replicates. Populations evolved from the *mutS* mutator genotype are in dark grey and resistant clones isolated from the WT ancestor are in light grey. All populations, except biofilm population 3 evolved from the *mutS* ancestor, significantly exceed WT resistance ($p < 0.05$, one-way ANOVA)

Extending the exposure to subinhibitory concentrations

The mutator genotype of *P. aeruginosa* facilitated the evolution of resistance to WLBU2 (Figure 1A), but isolating clones without other background mutations was challenging because of their increased mutation rate. Nonetheless, the parallelism in *morA*, *wspF* and *pmrB* is a strong indicator of the fitness benefits of these mutations (Cooper, 2018; Lieberman et al., 2011; Vogwill et al., 2014). As we found that resistance could be acquired by increasing the mutation supply, we tested whether increasing the number of generations in subinhibitory concentrations of WLBU2, thus increasing the mutations supplied before exposure to lethal concentrations, would increase the chance for the wild-type (non-mutator) ancestor of acquiring the mutations needed to survive the treatment.

169 We propagated five planktonic populations of wild-type *P. aeruginosa* PA14 for 10 days
170 under subinhibitory concentrations of WLBU2, followed by 2 days at inhibitory concentrations.
171 We estimate that the probability that any given nucleotide would be mutated during this regime is
172 0.74, with mean mutations per site of 1.52 (Table S2). Again, as a control, three populations were
173 propagated in the absence of WLBU2 to distinguish between broth and WLBU2 adaptive
174 mutations. Two populations survived the antibiotic treatment and showed higher levels of
175 resistance than the ancestor both to WLBU2 and to polymyxin B (Figures 1 and S1). Further, in
176 this study we detected different colony morphologies within each population at the end of the
177 experiment. Population 1 included small, rugose or wrinkly (Wr) colonies in addition to the smooth
178 (Sm) morphology of the ancestor, and population 2 contained both Sm and small (Sc) colonies. It
179 is interesting to note that small colony variants are associated with aggregation, increased biofilm
180 formation, and worse outcomes in chronic infections (Fux et al., 2005; Gloag et al., 2019). We
181 tested whether these clones produced more biofilm by measuring aggregation in the presence or
182 absence of WLBU2. We found that the Sc and Wr clones settled in clumps more than WT both in
183 absence and presence of WLBU2, while Sm colonies only clustered more in the presence of the
184 peptide (Figure 1B).
185 WGS of the two surviving populations and representative clones of each colony type
186 revealed that all acquired mutations in *pmrB* as well as mutations known to increase biofilm
187 production (Figure 2, Figure S2). The two small colony variants Wr and Sc acquired mutations in
188 *wspA* or *wspF* in the Wsp pathway seen previously in the mutator experiment, while one resistant
189 Sm clone acquired *orfN* and *pmrB* mutations as well as *yfiR*, another gene known to activate c-di-
190 GMP synthesis (Li et al., 2015). It is notable that these biofilm-activating mutations were selected
191 during planktonic propagation of the populations, suggesting that forming aggregates is a key step
192 for resistance to WLBU2. In addition, both resistant populations and the two clones with smooth
193 morphology had mutations in the *orfN* gene, which is involved in the synthesis of the O-antigen
194 (Rocchetta et al., 1999). Further, the Wr isolate had a mutation in *wbpM,* which also contributes
195 to O-antigen biosynthesis (Rocchetta et al., 1999). In the end, the only mutations common to both
196 the prolonged subinhibitory selection and the mutator lineages exposed to WLBU2 occurred in
197 *pmrB, orfN* and *wsp*, providing clear evidence of fitness benefits of these mutations affecting both
198 LPS composition and biofilm production in the presence of WLBU2 (Table 1).
199

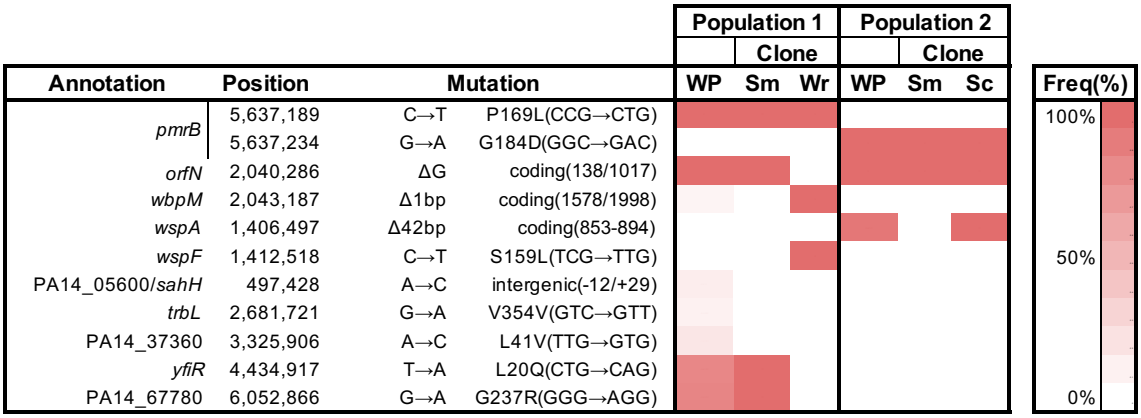

Figure 2. Mutations selected in the presence of subinhibitory WLBU2 and their frequencies. Each column shows the mutations present in one population or clone: WP = whole population; Sm and Wr are the smooth and wrinkly clones isolated from population 1; Sm and Sc are smooth and small colonies isolated from population 2. Only mutated genes that evolved in WLBU2-treated populations above 10% frequency found in the clones and not present in the non-WLBU2 controls are shown. A complete list of mutations is shown in Table S4.

		***mutS* strain**				**WT strain, subinhibitory treatment**					
		Biofilm		**Planktonic**		**Population 1**			**Population 2**		
Gene	**Pop1**	**Pop2**	**Pop3**	**Pop1**	**Pop2**	**Pop1**	**S**	**W**	**Pop2**	**S**	**Sc**
orfN	-	-	-	(10)G -> (9)G (138/1017 nt)	-	(10)G -> (9)G (138/1017 nt)	(10)G -> (9)G (138/1017 nt)	-	(10)G -> (9)G (138/1017 nt)	(10)G -> (9)G (138/1017 nt)	-
pmrB	V28L (59.7)	L180P (100)	L180P (49.3)	L18P (100)	L18P (31.0)	P169L (100)	P169L	P169L	G184D (100)	G184D	G184D
	L18P (17.3)		V28A (18.8)		V185A (20.5)						
	A248T (11.1)		D47G (15.8)		L296P (15.6)						
			F44L (8.5)		F408P (13.5)						
			L318P (7.1)								
wspF	Q282* (87.7)	L20P (24.2)	L20P (47.0)	L222P (31.2)	L51P (11.9)	-	-	S159L	-	-	-
				T274I (29.7)	L199P (7.8)						
				Q282* (14.3)	Q319 (6.6)						
					G283D (6.4)						
					G280D (5.3)						
					S159L (5.3)						
					Q299* (5.2)						

208 Table 1. Shared mutations selected across experiments. Each column shows the frequency (in
209 parentheses) of the mutations present in each population or clone. Only mutations evolved in the
210 populations exposed to WLBU2 above 5% frequency and not present in untreated controls are
211 shown.
212

213 **Discussion**

214 The effectiveness of the novel cationic peptide WLBU2 against a range of bacterial species
215 in multiple conditions and the absence of clear genetic mechanisms for resistance makes it a
216 promising candidate as a new antibiotic. However, one exception is its relative ineffectiveness
217 against the *Burkholderia cepacia* complex (Deslouches et al., 2015), which is generally resistant
218 to the cationic peptide polymyxin B. This unique result for these species suggests that multiple
219 modifications to LPS or outer envelope (e.g. associated with major species differences rather than
220 variation at a single locus) may be required for WLBU2 resistance. If multiple mutations are indeed
221 necessary for WLBU2 resistance, this should substantially lower the probability of evolved
222 resistance during treatment. This study provides strong support for this multiple mutation model,
223 but also shows that the pathway to resistance is plausible for mutator strains or ones already
224 containing one or more contributing mutations. Either possibility has been demonstrated in *P.*
225 *aeruginosa* infections: mutator isolates are frequently reported (Mena et al., 2008; Oliver et al.,
226 2000), mutations in the primary driver gene *pmrB,* mutations in the Wsp pathway, and mutations
227 affecting O-lipid biosynthesis are also common adaptations in clinical isolates that can be selected
228 for their advantages in biofilms even in the absence of antibiotics (Cannatelli et al., 2017; Gloag
229 et al., 2019; Moskowitz et al., 2012; Starkey et al., 2009).
230

231 It has been well demonstrated that resistance to cationic peptides in *P. aeruginosa*
232 involves modification of LPS (Fernández et al., 2013). Consistent with these findings, here we
233 detected parallel evolution in the *pmrB* and *orfN* genes across four independent lineages. PmrB
234 forms part of a two-component regulatory system that modifies the lipid-A composition, a
235 mechanism that is commonly associated with cationic peptide resistance in several species
236 (Cannatelli et al., 2017; Moskowitz et al., 2012). In combination with *pmrB* variants, mutations
237 in *orfN* and *wbpM* were found in WBLU2-resistant populations and clones. OrfN and WbpM
238 form part of the operon that synthetizes the LPS O-antigen, and mutations in *orfN* are predicted
239 to increase antibiotic resistance by reducing membrane permeability (Rocchetta et al., 1999;

240 Scribner et al., 2020). Furthermore, we observed mutations in *wspA*, *wspF*, and *yfiR* that increase
241 aggregation and/or biofilm production, likely through increased cyclic-di-GMP that in turn
242 increases production of the cationic Pel polysaccharide. Mutations in the phosphodiesterase
243 domain of *morA* also were repeatedly selected in mutator populations with a likely similar effect
244 on cyclic-di-GMP and biofilm (Table S3). Aggregation by secretion of a biofilm polymer is
245 therefore another likely mechanism of WLBU2 resistance or tolerance (Gloag et al., 2019;
246 Jennings et al., 2015; Starkey et al., 2009).
247 As AMR spreads around the globe, it is crucial to develop new antibiotics so that we still
248 have treatments available for otherwise routine infections. When considering the development of
249 new antibiotics, it is necessary to anticipate the possible mechanisms of resistance so that
250 treatments can be designed more effectively to counteract the emergence of resistance (Brockhurst
251 et al., 2019; MacLean, 2020). We often read claims of new 'evolution-proof' antibiotics (Ling et
252 al., 2015; Zasloff, 2002), but the absence of resistance mechanisms can be due to low mutation
253 supply as observed in our experimental approach or because of the lack of genetic diversity among
254 sampled strains (Bell and MacLean, 2018). Here, we described the genetic causes of evolved
255 resistance to the promising new cationic peptide WLBU2 by tackling both of the issues.
256 Combining experimental evolution and whole population genome sequencing, it appears likely
257 that WLBU2 has the advantageous property of requiring two or more mutations that affect the
258 outer membrane as well as aggregation for resistance to evolve. Further biochemical assays are
259 needed to determine the actual role of each mutation in the resistance phenotype. This additional
260 research notwithstanding, we believe that effort should be spent elucidating the evolutionary
261 pathways driving antibiotic resistance when developing antibiotics, because knowing likely
262 adaptations could help in designing more accurate treatments and limit the spread of AMR,
263 preserving the longevity of the antibiotic in clinical use.
264

265 **Methods**

266 **Experimental evolution**

267 We performed three independent evolution experiments. In all of them, we propagated the
268 ancestor strains in a modified M9 medium (referred to as $M9^+$) containing 0.37 mM $CaCl_2$, 8.7
269 mM $MgSO_4$, 42.2 mM Na_2HPO_4, 22 mM KH2PO_4, 21.7mM NaCl, 18.7 mM NH_4Cl and 0.2 g/L
270 glucose and supplemented with 20 mL/L MEM essential amino acids (Gibco 11130051), 10 mL/L
271 MEM nonessential amino acids (Gibco 11140050), and 10 mL each of trace mineral solutions A,
272 B, and C (Corning 25021-3Cl).

273 In the first experiment, we selected a single clone of *A. baumannii* ATCC17978 and *P.*
274 *aeruginosa* PA14 and propagated them independently for 24 hours in $M9^+$ in the absence of
275 antibiotic. We then sub-cultured each population into 10 replicate populations. Ten of the
276 populations (5 planktonic and 5 biofilm) were propagated every 24 hours in increasing
277 concentrations of WLBU2 starting at 0.5X the minimum inhibitory concentration (MIC). We
278 doubled the WLBU2 concentrations after 72 hours until no population survived the treatment. As
279 a control, we propagated the same number of strains both planktonically and in biofilm in the
280 absence of antibiotic. We also propagated 10 populations (5 biofilm and 5 planktonic) in increasing
281 concentration of the cationic peptide polymyxin B.

282 In the second experiment, we propagated 5 planktonic populations and 5 biofilm
283 populations under the same conditions described above, a mutator strain of *P. aeruginosa* obtained
284 from (Flynn et al., 2016). We also propagated 3 planktonic populations and 3 biofilm populations
285 of the hypermutant strain in the absence of antibiotic pressure.

286 In the third experiment, we propagated 5 planktonic populations of the non-hypermutant
287 laboratorial strain *P. aeruginosa* PA14 under subinhibitory concentrations of WLBU2 for 10 days.
288 Then, we propagated the populations that survived the treatment with 1X MIC of WLBU2 2 days.

289 In all three experiments, we froze 1mL of the surviving populations at days 1, 3, 4, 6, 7, 9,
290 10, and 12 in 9% of DMSO (before and after increases in antibiotics.)

291 **Phenotypic characterization: antimicrobial susceptibility and aggregation assay**

292 We determined the MIC to WLBU2 and polymyxin B of the whole populations and 4
293 clones by broth microdilution according to the Clinical and Laboratory Standards Institute
294 guidelines (CLSI, 2019), in which each bacterial sample was tested in 2-fold-increasing

295 concentrations of each antibiotic. WLBU2 was provided by Peptilogics (Peptilogics, Pittsburgh,
296 PA) and polymyxin B was provided by Alfa Aesar (Alfa Aesar, Wardhill, MA).
297 To determine the aggregation ability of the 4 clones selected in the subinhibitory
298 experiment, we grew two replicates (R1 and R2) of the 4 clones and the ancestral strain in 5ml of
299 M9+ for 24 hours at 37ºC in a roller drum at 200 RPM. After 24 hours we transferred the whole
300 culture of each clone to a 13 mm glass tube, and let the tubes settle over 24 hours at 4ºC without
301 shaking. We vortexed replicate 1 (R1) of each clone for 30s and measured the OD_{600}. From R2,
302 we carefully took 200 µl of the top part of the tube without vortexing and measured the OD_{600}. The
303 aggregation percentage was estimated as 100(1 - OD_{600} R2 / 1 – OD_{600} R1). We performed this
304 experiment with five replicates both in the absence and presence (4 µg/ml) of WLBU2.
305 **Genome sequencing**
306 We performed whole population genome sequencing of population that survived the
307 treatment in each experiment as well as control lines. This includes 3 biofilm and 2 planktonic
308 populations of mutator populations surviving WLBU2 treatment and 4 biofilm and 3 planktonic
309 populations evolved in the absence of the antibiotic (12 total populations). In addition, we
310 sequenced the 2 populations and 4 clones that survived the subinhibitory WLBU2 treatment, as
311 well as one untreated control population (2 populations, 4 clones). Finally, we sequenced 3 biofilm
312 and 3 planktonic populations of *A. baumannii* that were propagated in presence of polymyxin B
313 as well 3 three planktonic and 3 biofilm populations (12 total populations).
314 We revived each population or clone from a freezer stock in the growth conditions under
315 which they were isolated and grew for 24 hours. DNA was extracted using the Qiagen DNAeasy
316 Blood and Tissue kit (Qiagen, Hiden, Germany). The sequencing library was prepared as described
317 by Turner and colleagues (Turner et al., 2018) according to the protocol of Baym *et al.* (Baym et
318 al., 2015), using the Illumina Nextera kit (Illumina Inc., San Diego, CA) and sequenced using an
319 Illumina NextSeq500 at the Microbial Genome Sequencing center (www.migscenter.com).
320 **Data processing**
321 The variants were called using the breseq software package v0.31.0 (Barrick et al., 2014)
322 using the default parameters and the -p flag when required for identifying polymorphisms in
323 populations after all sequences were first quality filtered and trimmed with the Trimmomatic
324 software v0.36 (Bolger et al., 2014) using the criteria: LEADING:20 TRAILING:20
325 SLIDINGWINDOW:4:20 MINLEN:70. The version of *A. baumannii* ATCC 17978-mff

326 (GCF_001077675.1 downloaded from the NCBI RefSeq database,17-Mar-2017) was used as the
327 reference genome for variant calling. We added the two additional plasmid sequences present in
328 the *A. baumannii* strain (NC009083, NC_009084) to the chromosome NZ_CP012004 and plasmid
329 NZ_CP012005. The version of *P. aeruginosa* UCBPP-PA14 was downloaded from RefSeq on 25-
330 Aug-2020. To remove false positives using the following strategy: mutations were removed if they
331 never reached a cumulative frequency across time points of 25%, or if mutations were also found
332 in the ancestor's genome. The *mutS* clone used in this study is a merodiploid bearing both the WT
333 and the mutated *mutS* gene (Flynn et al., 2016). As breseq can fail to detect mutations when
334 analyzing repeated sequences (Barrick et al., 2014), we manually visualized the *mutS* locus with
335 the bam files generated by breseq using the Integrative genomics viewer software, confirming that
336 the mutation was present in the ancestral clone and in all evolved populations sequenced at *ca.*
337 50% frequency (Robinson et al., 2011).

338 **Data Availability**

339 R code for filtering and data processing are deposited here:
340 https://github.com/sirmicrobe/U01_allele_freq_code/tree/master/wlbu2_paper. All sequences
341 were deposited into NCBI under the Bioproject accession number PRJNA663022.

342 **Acknowledgments**

343 We thank Jonathan Steckbeck, Ken Urish and Peptidologics for kindly providing WLBU2
344 and for their technical assistance. We thank Jeronimo Rodriguez-Beltran and Cristina Herencias
345 for proofreading of the paper. We also thank Dan Snyder and MiGS (www.migscenter.com) for
346 whole genome sequencing. This research was supported by NIH U01AI124302-01 and, in part,
347 under PA CURES Grant #4100085725 with the Pennsylvania Department of Health. The
348 Department specifically disclaims responsibility for any analyses, interpretations or conclusions.

349
350
351
352
353
354
355

356 **References**

357

358 Ahmed, M.N., Porse, A., Sommer, M.O.A., Hoiby, N., and Ciofu, O. (2018). Evolution of
359 antibiotic resistance in biofilm and planktonic P. aeruginosa populations exposed to sub-
360 inhibitory levels of ciprofloxacin. Antimicrobial Agents and Chemotherapy.

361 Bailey, S.F., Rodrigue, N., and Kassen, R. (2015). The effect of selection environment on the
362 probability of parallel evolution. Molecular Biology and Evolution *32*, 1436–1448.

363 Barbosa, C., Römhild, R., Rosenstiel, P., and Schulenburg, H. (2019). Evolutionary stability of
364 collateral sensitivity to antibiotics in the model pathogen Pseudomonas aeruginosa. ELife *8*,
365 e51481.

366 Barrick, J.E., Colburn, G., Deatherage, D.E., Traverse, C.C., Strand, M.D., Borges, J.J.,
367 Knoester, D.B., Reba, A., and Meyer, A.G. (2014). Identifying structural variation in haploid
368 microbial genomes from short-read resequencing data using breseq. BMC Genomics *15*, 1039.

369 Baym, M., Kryazhimskiy, S., Lieberman, T.D., Chung, H., Desai, M.M., and Kishony, R.
370 (2015). Inexpensive multiplexed library preparation for megabase-sized genomes. PLoS One *10*,
371 e0128036.

372 Bell, G., and MacLean, C. (2018). The Search for 'Evolution-Proof' Antibiotics. Trends in
373 Microbiology *26*, 471–483.

374 Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina
375 sequence data. Bioinformatics *30*, 2114–2120.

376 Brockhurst, M.A., Harrison, F., Veening, J.W., Harrison, E., Blackwell, G., Iqbal, Z., and
377 Maclean, C. (2019). Assessing evolutionary risks of resistance for new antimicrobial therapies.
378 Nature Ecology & Evolution *3*, 515–517.

379 Cannatelli, A., Giani, T., Aiezza, N., Di Pilato, V., Principe, L., Luzzaro, F., Galeotti, C.L., and
380 Rossolini, G.M. (2017). An allelic variant of the PmrB sensor kinase responsible for colistin
381 resistance in an Escherichia coli strain of clinical origin. Scientific Reports *7*, 1–6.

382 Chen, C., Deslouches, B., Montelaro, R.C., and Di, Y.P. (2018). Enhanced efficacy of the
383 engineered antimicrobial peptide WLBU2 via direct airway delivery in a murine model of P.
384 aeruginosa pneumonia. Clin Microbiol Infect *24*, 547.e1-547.e8.

385 CLSI (2019). Performance Standards for Antimicrobial Susceptibility Testing (Wayne, PA:
386 Clinical and Laboratory Standards Institute).

387 Cooper, V.S. (2018). Experimental Evolution as a High-Throughput Screen for Genetic
388 Adaptations. MSphere *3*.

389 Deslouches, B., Steckbeck, J.D., Craigo, J.K., Doi, Y., Burns, J.L., and Montelaro, R.C. (2015).
390 Engineered Cationic Antimicrobial Peptides To Overcome Multidrug Resistance by ESKAPE
391 Pathogens. Antimicrob Agents Chemother *59*, 1329–1333.

392 Di, Y.P., Lin, Q., Chen, C., Montelaro, R.C., Doi, Y., and Deslouches, B. (2020). Enhanced
393 therapeutic index of an antimicrobial peptide in mice by increasing safety and activity against
394 multidrug-resistant bacteria. Sci Adv *6*, eaay6817.

395 Dillon, M.M., Sung, W., Sebra, R., Lynch, M., and Cooper, V.S. (2017). Genome-Wide Biases
396 in the Rate and Molecular Spectrum of Spontaneous Mutations in Vibrio cholerae and Vibrio
397 fischeri. Molecular Biology and Evolution *34*, 93–109.

398 Fernández, L., Álvarez-Ortega, C., Wiegand, I., Olivares, J., Kocíncová, D., Lam, J.S., Martínez,
399 J.L., and Hancock, R.E.W. (2013). Characterization of the Polymyxin B Resistome of
400 Pseudomonas aeruginosa. Antimicrobial Agents and Chemotherapy *57*, 110–119.

401 Flynn, K.M., Dowell, G., Johnson, T.M., Koestler, B.J., Waters, C.M., and Cooper, V.S. (2016).
402 Evolution of Ecological Diversity in Biofilms of Pseudomonas aeruginosa by Altered Cyclic
403 Diguanylate Signaling. J. Bacteriol. *198*, 2608–2618.

404 Fux, C.A., Costerton, J.W., Stewart, P.S., and Stoodley, P. (2005). Survival strategies of
405 infectious biofilms. Trends in Microbiology *13*, 34–40.

406 Gifford, D.R., Berríos-Caro, E., Joerres, C., Galla, T., and Knight, C.G. (2019). Mutators drive
407 evolution of multi-resistance to antibiotics. BioRxiv 643585.

408 Gloag, E.S., Marshall, C.W., Snyder, D., Lewin, G.R., Harris, J.S., Santos-Lopez, A., Chaney,
409 S.B., Whiteley, M., Cooper, V.S., and Wozniak, D.J. (2019). Pseudomonas aeruginosa
410 Interstrain Dynamics and Selection of Hyperbiofilm Mutants during a Chronic Infection. MBio
411 *10*, e01698-19.

412 Gullberg, E., Cao, S., Berg, O.G., Ilbäck, C., Sandegren, L., Hughes, D., and Andersson, D.I.
413 (2011). Selection of Resistant Bacteria at Very Low Antibiotic Concentrations. PLoS Pathogens
414 *7*, e1002158.

415 Ha, D.-G., and O'Toole, G.A. (2015). c-di-GMP and its Effects on Biofilm Formation and
416 Dispersion: a Pseudomonas Aeruginosa Review. Microbiology Spectrum *3*.

417 Hernando-Amado, S., Sanz-Garcia, F., and Martinez, J.L. (2019). Antibiotic resistance evolution
418 is contingent on the quorum sensing response in Pseudomonas aeruginosa. Molecular Biology
419 and Evolution.

420 Hickman, J.W., Tifrea, D.F., and Harwood, C.S. (2005). A chemosensory system that regulates
421 biofilm formation through modulation of cyclic diguanylate levels. PNAS *102*, 14422–14427.

422 Huangyutitham, V., Güvener, Z.T., and Harwood, C.S. (2013). Subcellular clustering of the
423 phosphorylated WspR response regulator protein stimulates its diguanylate cyclase activity.
424 MBio *4*, e00242-00213.

425 Hughes, D., and Andersson, D.I. (2017). Evolutionary Trajectories to Antibiotic Resistance.
426 Annual Review of Microbiology *71*, 579–596.

427 Ibacache-Quiroga, C., Oliveros, J.C., Couce, A., and Blázquez, J. (2018). Parallel Evolution of
428 High-Level Aminoglycoside Resistance in Escherichia coli Under Low and High Mutation
429 Supply Rates. Front. Microbiol. *9*.

430 Jennings, L.K., Storek, K.M., Ledvina, H.E., Coulon, C., Marmont, L.S., Sadovskaya, I., Secor,
431 P.R., Tseng, B.S., Scian, M., Filloux, A., et al. (2015). Pel is a cationic exopolysaccharide that
432 cross-links extracellular DNA in the Pseudomonas aeruginosa biofilm matrix. PNAS *112*,
433 11353–11358.

434 Kumagai, A., Dupuy, F.G., Arsov, Z., Elhady, Y., Moody, D., Ernst, R.K., Deslouches, B.,
435 Montelaro, R.C., Di, Y.P., and Tristram-Nagle, S. (2019). Elastic behavior of model membranes
436 with antimicrobial peptides depends on lipid specificity and D-enantiomers. Soft Matter *15*,
437 1860–1868.

438 Lashua, L.P., Melvin, J.A., Deslouches, B., Pilewski, J.M., Montelaro, R.C., and Bomberger,
439 J.M. (2016). Engineered cationic antimicrobial peptide (eCAP) prevents Pseudomonas
440 aeruginosa biofilm growth on airway epithelial cells. J Antimicrob Chemother *71*, 2200–2207.

441 Li, S., Li, T., Xu, Y., Zhang, Q., Zhang, W., Che, S., Liu, R., Wang, Y., and Bartlam, M. (2015).
442 Structural insights into YfiR sequestering by YfiB in Pseudomonas aeruginosa PAO1. Scientific
443 Reports *5*, 16915.

444 Lieberman, T.D., Michel, J.B., Aingaran, M., Potter-Bynoe, G., Roux, D., Davis, M.R., Skurnik,
445 D., Leiby, N., LiPuma, J.J., Goldberg, J.B., et al. (2011). Parallel bacterial evolution within
446 multiple patients identifies candidate pathogenicity genes. Nat Genet *43*, 1275–1280.

447 Lin, Q., Deslouches, B., Montelaro, R.C., and Di, Y.P. (2018). Prevention of ESKAPE pathogen
448 biofilm formation by antimicrobial peptides WLBU2 and LL37. Int. J. Antimicrob. Agents *52*,
449 667–672.

450 Ling, L.L., Schneider, T., Peoples, A.J., Spoering, A.L., Engels, I., Conlon, B.P., Mueller, A.,
451 Schäberle, T.F., Hughes, D.E., Epstein, S., et al. (2015). A new antibiotic kills pathogens without
452 detectable resistance. Nature *517*, 455–459.

453 MacLean, R.C. (2020). Assessing the Potential for Staphylococcus aureus to Evolve Resistance
454 to XF-73. Trends in Microbiology *0*.

455 MacLean, R.C., and San Millan, A. (2019). The evolution of antibiotic resistance. Science *365*,
456 1082–1083.

457 Maltas, J., and Wood, K.B. (2019). Pervasive and diverse collateral sensitivity profiles inform
458 optimal strategies to limit antibiotic resistance. PLOS Biology *17*, e3000515.

459 Mandell, J.B., Deslouches, B., Montelaro, R.C., Shanks, R.M.Q., Doi, Y., and Urish, K.L.
460 (2017). Elimination of Antibiotic Resistant Surgical Implant Biofilms Using an Engineered
461 Cationic Amphipathic Peptide WLBU2. Sci Rep *7*.

462 Mandell, J.B., Koch, J.A., Deslouches, B., and Urish, K.L. (2020). Direct antimicrobial activity
463 of cationic amphipathic peptide WLBU2 against Staphylococcus aureus biofilms is enhanced in
464 physiologic buffered saline. Journal of Orthopaedic Research.

465 Mehta, H.H., Prater, A.G., and Shamoo, Y. (2018). Using experimental evolution to identify
466 druggable targets that could inhibit the evolution of antimicrobial resistance. The Journal of
467 Antibiotics *71*, 279–286.

468 Melvin, J.A., Lashua, L.P., Kiedrowski, M.R., Yang, G., Deslouches, B., Montelaro, R.C., and
469 Bomberger, J.M. (2016). Simultaneous Antibiofilm and Antiviral Activities of an Engineered
470 Antimicrobial Peptide during Virus-Bacterium Coinfection. MSphere *1*.

471 Mena, A., Smith, E.E., Burns, J.L., Speert, D.P., Moskowitz, S.M., Perez, J.L., and Oliver, A.
472 (2008). Genetic Adaptation of Pseudomonas aeruginosa to the Airways of Cystic Fibrosis
473 Patients Is Catalyzed by Hypermutation. Journal of Bacteriology *190*, 7910–7917.

474 Moskowitz, S.M., Brannon, M.K., Dasgupta, N., Pier, M., Sgambati, N., Miller, A.K., Selgrade,
475 S.E., Miller, S.I., Denton, M., Conway, S.P., et al. (2012). PmrB Mutations Promote Polymyxin
476 Resistance of Pseudomonas aeruginosa Isolated from Colistin-Treated Cystic Fibrosis Patients.
477 Antimicrob Agents Chemother *56*, 1019–1030.

478 Oliver, A., Cantón, R., Campo, P., Baquero, F., and Blázquez, J. (2000). High Frequency of
479 Hypermutable Pseudomonas aeruginosa in Cystic Fibrosis Lung Infection. Science *288*, 1251–
480 1253.

481 Papkou, A., Hedge, J., Kapel, N., Young, B., and MacLean, R.C. (2020). Efflux pump activity
482 potentiates the evolution of antibiotic resistance across S. aureus isolates. Nature
483 Communications *11*, 3970.

484 Paranjape, S.M., Lauer, T.W., Montelaro, R.C., Mietzner, T.A., and Vij, N. (2013). Modulation
485 of proinflammatory activity by the engineered cationic antimicrobial peptide WLBU-2.
486 F1000Res *2*, 36.

487 Perron, G.G., Zasloff, M., and Bell, G. (2006). Experimental evolution of resistance to an
488 antimicrobial peptide. Proc Biol Sci *273*, 251–256.

489 Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and
490 Mesirov, J.P. (2011). Integrative genomics viewer. Nature Biotechnology *29*, 24–26.

491 Rocchetta, H.L., Burrows, L.L., and Lam, J.S. (1999). Genetics of O-Antigen Biosynthesis in
492 Pseudomonas aeruginosa. Microbiol Mol Biol Rev *63*, 523–553.

493 Santos-Lopez, A., Marshall, C.W., Scribner, M.R., Snyder, D.J., and Cooper, V.S. (2019).
494 Evolutionary pathways to antibiotic resistance are dependent upon environmental structure and
495 bacterial lifestyle. ELife *8*, e47612.

496 Santos-Lopez, A., Marshall, C.W., Welp, A.L., Turner, C.B., Rasero, J., and Cooper, V.S.
497 (2020). The roles of history, chance, and natural selection in the evolution of antibiotic
498 resistance. BioRxiv 2020.07.22.216465.

499 Scribner, M.R., Santos-Lopez, A., Marshall, C.W., Deitrick, C., and Cooper, V.S. (2020).
500 Parallel Evolution of Tobramycin Resistance across Species and Environments. MBio *11*.

501 Shaver, A.C., Dombrowski, P.G., Sweeney, J.Y., Treis, T., Zappala, R.M., and Sniegowski, P.D.
502 (2002). Fitness Evolution and the Rise of Mutator Alleles in Experimental Escherichia coli
503 Populations. Genetics *162*, 557–566.

504 Spohn, R., Daruka, L., Lázár, V., Martins, A., Vidovics, F., Grézal, G., Méhi, O., Kintses, B.,
505 Számel, M., Jangir, P.K., et al. (2019). Integrated evolutionary analysis reveals antimicrobial
506 peptides with limited resistance. Nature Communications *10*, 1–13.

507 Starkey, M., Hickman, J.H., Ma, L., Zhang, N., Long, S.D., Hinz, A., Palacios, S., Manoil, C.,
508 Kirisits, M.J., Starner, T.D., et al. (2009). Pseudomonas aeruginosa Rugose Small-Colony
509 Variants Have Adaptations That Likely Promote Persistence in the Cystic Fibrosis Lung. Journal
510 of Bacteriology *191*, 3492–3503.

511 Swedan, S., Shubair, Z., and Almaaytah, A. (2019). Synergism of cationic antimicrobial peptide
512 WLBU2 with antibacterial agents against biofilms of multi-drug resistant Acinetobacter
513 baumannii and Klebsiella pneumoniae. Infect Drug Resist *12*, 2019–2030.

514 Toprak, E., Veres, A., Michel, J.B., Chait, R., Hartl, D.L., and Kishony, R. (2011). Evolutionary
515 paths to antibiotic resistance under dynamically sustained drug selection. Nat Genet *44*, 101–
516 105.

517 Trampari, E., Holden, E.R., Wickham, G.J., Ravi, A., Prischi, F., Martins, L. de O., Savva, G.M.,
518 Bavro, V.N., and Webber, M.A. (2019). Antibiotics select for novel pathways of resistance in
519 biofilms. BioRxiv 605212.

520 Turner, C.B., Marshall, C.W., and Cooper, V.S. (2018). Parallel genetic adaptation across
521 environments differing in mode of growth or resource availability. Evolution Letters *2*, 355–367.

522 de Visser, A.J.G.M., Zeyl, C.W., Gerrish, P.J., Blanchard, J.L., and Lenski, R.E. (1999).
523 Diminishing Returns from Mutation Supply Rate in Asexual Populations. Science *283*, 404–406.

524 Vogwill, T., Kojadinovic, M., Furio, V., and MacLean, R.C. (2014). Testing the role of genetic
525 background in parallel evolution using the comparative experimental evolution of antibiotic
526 resistance. Molecular Biology and Evolution *31*, 3314–3323.

527 Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. Nature *415*, 389–395.

528

529 **Table S1.** Mutations selected in the presence of increasing concentrations of polymyxin B. Two
530 sheets can be found in the Table S1: "PolymyxinB_raw" which includes all mutations called by
531 breseq and "PolymyxinB_relevant" which include the mutations selected for in presence of
532 polymyxin B but not selected in the absence of the antibiotic.
533
534 **Table S2.** Estimated mutation probabilities during experimental evolution.
535
536 **Table S3.** Mutations selected using the *mutS* deficient strain. Three sheets can be found in the
537 document: "raw_mutations" include all mutations called by breseq, "no_controls_>0.1" include
538 the cured list of mutations subtracting from the raw list all mutations happening in the populations
539 evolving without WLBU2 and with lower frequency than 0.1, and "parallel_genes" which include
540 the three genes that are targeted in more than one resistant population and are not present in any
541 control.
542
543 **Table S4.** Mutations selected in the subinhibitory experiment. Two sheets can be found in the
544 document: "populations" and "clones" include all mutations called by breseq in the evolving
545 populations and clones respectively. Note that the curated list of mutations, subtracting those found
546 in controls without antibiotic and the ancestral strain, is listed in Figure 2.
547