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Main Manuscript for

The Evolution of Heteroresistance via Small Colony Variants in *Escherichia coli* Following Long Term Exposure to Bacteriostatic Antibiotics

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Investigation: ICM, APS, JAM, TGG, BAB
Visualization: JAM, TGG
Funding Acquisition: BRL
Project Administration: BRL
Supervision: BRL
Writing– Initial Draft: BRL, FB, APS, JAM, TGG, BAB
Writing– Review & Editing: BRL, FB, ICM, APS, JAM, TGG, BAB

Competing Interest Statement: The authors have no competing interests.

Keywords: Heteroresistance, antibiotic resistance, bacteriostatic antibiotics, evolution, small colony variants, Escherichia coli

This PDF file includes:

- Main Text
- Figures 1 to 5
- References

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1 **Abstract**
2 Traditionally, bacteriostatic antibiotics are agents able to arrest bacterial growth. Despite
3 being unable to kill bacterial cells, when they are used clinically the outcome of these drugs
4 is frequently as effective as when a bactericidal drug is used. We explore the dynamics of
5 *Escherichia coli* after exposure to two ribosome-targeting bacteriostatic antibiotics,
6 chloramphenicol and azithromycin, for thirty days. The results of our experiments provide
7 evidence that bacteria exposed to these drugs replicate, evolve, and generate a sub-
8 population of small colony variants (SCVs) which are resistant to multiple drugs. These
9 SCVs contribute to the evolution of heteroresistance and rapidly revert to a susceptible
10 state once the antibiotic is removed. Stated another way, exposure to bacteriostatic drugs
11 selects for the evolution of heteroresistance in populations previously lacking this trait.
12 More generally, our results question the definition of bacteriostasis as populations exposed
13 to bacteriostatic drugs are replicating despite the lack of net growth.

14
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16 **Main Text**
17
18 **Introduction**
19
20 Antibiotics can be broadly classified as being bactericidal or bacteriostatic based on
21 whether they kill bacteria or simply arrest their growth [1]. Intuitively, it would make sense
22 to treat an infection with drugs that kill the infecting bacteria, the bactericidal drugs, and
23 thereby eliminate the reliance on the host's immune system to clear the infection, as would
24 be the case with bacteriostatic antibiotics. For this reason, bacteriostatic drugs have been
25 considered "weaker" than bactericidal drugs and are not recommended for the treatment of
26 severe infections or infections in immunodeficient patients [2,3].
27
28 This distinction between bactericidal and bacteriostatic drugs is manifest in quantitative
29 experimental studies of the pharmacodynamics (PD) of antibiotics and bacteria. These
30 studies focus on the rates and dynamics of the drug's ability to kill exposed populations of
31 bacteria [4-7]. Many of the studies concerning why antibiotics fail to control bacterial
32 infections have focused on phenomena solely belonging to bactericidal antibiotics such as
33 persistence and tolerance [6,8-10]. With much of the clinical application of antibiotics focusing
34 on bactericidal drugs and the majority of the research on the PD also focusing on
35 bactericidal drugs, PD research on bacteriostatic drugs has been relatively neglected.
36
37 However, in recent years, clinicians have given less importance to the antibiotic's ability
38 to kill bacteria *in vitro* and instead have focused on the outcome of treatment with these
39 drugs. By this criterion, in many cases bacteriostatic antibiotics are as effective as
40 bactericidal even in severe infections, with the possible exception of immunosuppressed
41 patients [2,11]. The increase in use of bacteriostatic antibiotics could reduce the selection
42 pressure for resistance to bactericidal agents used in these critical infections. However, the
43 shift to using bacteriostatic antibiotics requires the development of quantitative measures
44 of the PD of antibiotics that arrest the growth of, rather than kill, bacteria [12]. One cannot
45 solely characterize the PD of bacteriostatic antibiotics by the minimum concentration
46 required to prevent the replication of exposed bacteria, the MIC. Furthermore, the evolution
47 of genomic resistance for these agents is rare and the resistant traits tolerance or persistence
48 that occur in bactericidal antibiotics are difficult to define and perhaps impossible to detect
49 with these bacteriostatic agents. This raises questions about the population and
50 evolutionary dynamics of bacteria confronted with these drugs. If the bacteria exposed to
51 bacteriostatic antibiotics are not replicating, one would not expect them to evolve.
52 Therefore, more considerations of the pharmaco-, population, and evolutionary dynamics
53 of bacteriostatic antibiotics are needed both academically and clinically.
54
55 In this study, we explore the pharmaco-, population, and evolutionary dynamics of
56 *Escherichia coli* exposed to two bacteriostatic antibiotics of different classes,
57 chloramphenicol (CHL) and azithromycin (AZM), over 30 days. The results of our
58 experiments provide evidence that: (i) long term exposure to these ribosome-targeting
59 bacteriostatic antibiotics does not change the absolute density of exposed populations, (ii)
60 despite the fact that the population's net density does not change, bacteria exposed to these
61 drugs replicate, evolve, and generate small colony variants, and (iii) the selective pressure

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62 mediated by these drugs favors the evolution of heteroresistant populations, i.e. the
63 emergence of resistant minority populations, in a strain previously lacking this trait.
64
65
66

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67 **Results**
68
69 **Long Term Exposure to Bacteriostatic Antibiotics**
70
71 We begin our investigation into the effects of long term exposure to ribosome-targeting
72 bacteriostatic drugs by evaluating the impact that these agents have on bacterial survival
73 over 30 days. We exposed four independent cultures of ~10^5 CFU/mL of *E. coli* MG1655
74 in glucose-limited minimal media to super-MIC (Minimum Inhibitory Concentration)
75 concentrations of CHL and AZM for 30 days (Figure 1). The MIC of CHL and AZM with
76 MG1655 were estimated by broth microdilution in this glucose-limited minimal media and
77 found to be 6.25 ug/mL for both drugs [13]. Super-MIC concentrations of each drug that were
78 shown to be bacteriostatic with minimal killing and/or growth were used in the experiment
79 (Supplemental Figure 1). Cultures exposed to these drugs were sampled every 5 days. We
80 observed that over the course of the experiment, the control cultures containing no drugs
81 reached its maximum stationary phase density of approximately 10^9 CFU/mL and then
82 went down by approximately two logs over the course of 30 days, while the densities in
83 the cultures containing the drugs remained stable, with at most a half-log change in density
84 in the drug-treated populations. Notably, in drug-treated cultures where the bacteria were
85 not lost, two distinct colony morphologies emerged. The colonies were either similar to the
86 ancestral wild-type *E. coli* or were much smaller bacterial colonies, small colony variants
87 (SCVs). This evolution occurred while under strong selective pressure from these drugs.
88 There was no change in colony size in the drug-free controls. To assess the maintenance of
89 the activity of the antibiotics after 30 days, bacteria resistant to the antibiotic in each culture
90 was added at approximately 10^6 CFU/mL and over the course of 24 hours each culture
91 grew approximately three orders of magnitude (Supplemental Figure 2). This residual
92 growth indicates that at 30 days the antibiotic is still at a super-MIC concentration and
93 thereby is the limiting factor for growth in the long term experiments.
94
95 To determine how evolution is occurring in the apparent absence of net growth in the
96 presence of the antibiotic, we performed a long term experiment using a conditionally non-
97 replicative plasmid [14] to identify if growth is occurring at the same rate as death (Figure 2).
98 After 10 days the plasmid frequency decreased by 100-fold, and after 20 days the plasmid
99 frequency was near the limit of detection. That means that the plasmid containing cells are
100 progressively diluted (as each cell division gives rise to a plasmid-free descendant) and
101 indicates that the population is growing and replicating at least once a day and dying at the
102 same rate.
103
104

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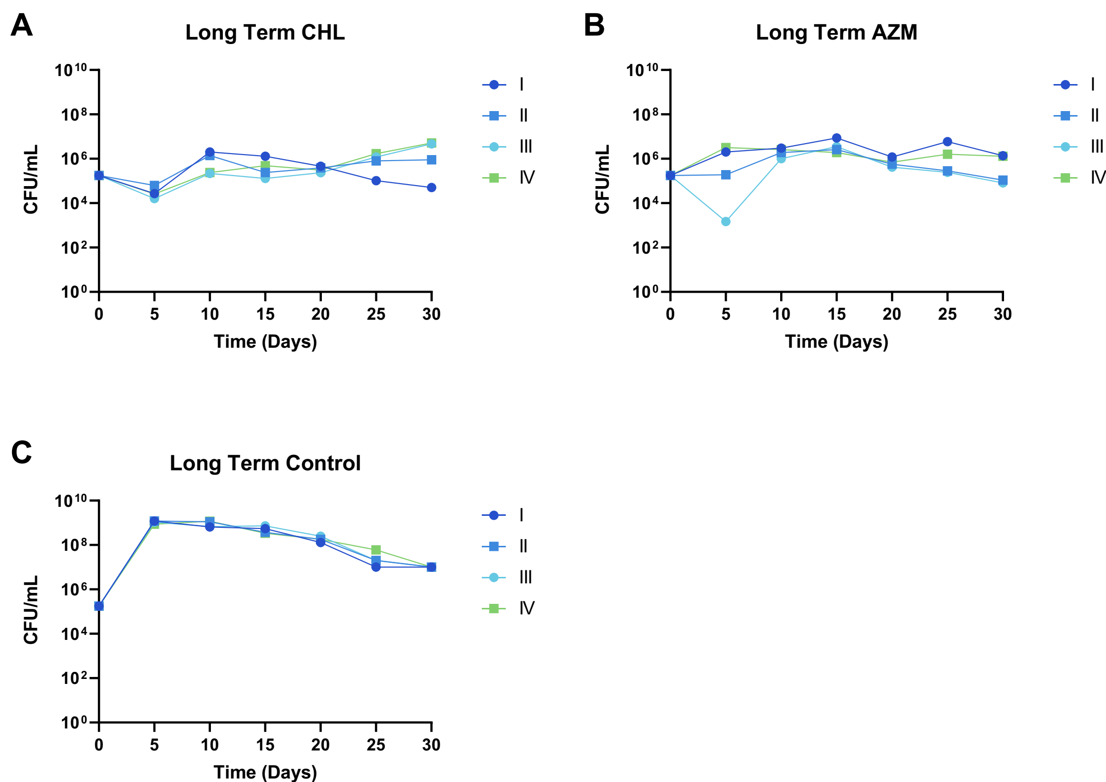
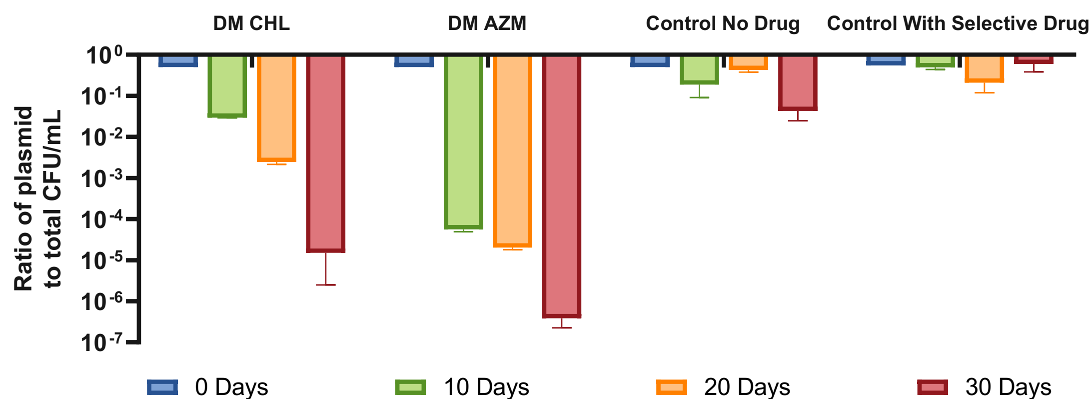


105
106 **Fig 1. Long term exposure of *E. coli* to bacteriostatic drugs.** Density in CFU/mL of *E.*
107 *coli* MG1655 measured every 5 days for 30 days of 4 independent biological replicas (I-
108 IV). **(A)** *E. coli* exposed to 4x MIC CHL; **(B)** *E. coli* exposed to 3x MIC AZM; **(C)** Drug-
109 free control.
110



111

112 **Fig 2. Long term experiment with a non-replicative plasmid.** Ratio of the plasmid-
113 containing cells to the total number of cells in CFU/mL of *E. coli* MG1655 with the non-
114 replicative plasmid pAM34 which bears an ampicillin resistance cassette. The total cell
115 density and density of cells bearing the plasmid were measured every 10 days for 30 days
116 of 4 independent biological replicas in four conditions: i) minimal media with CHL, ii)

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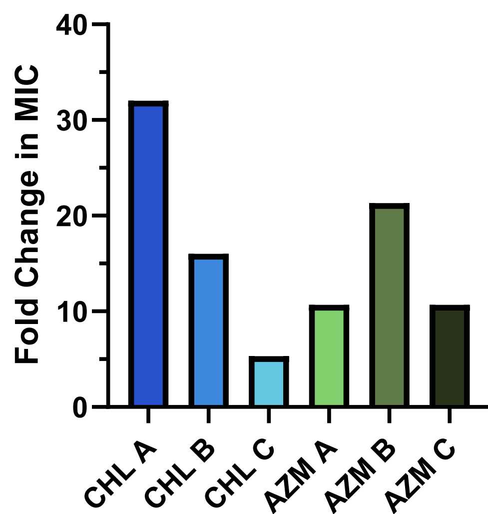
117 minimal media with AZM, iii) minimal media with no antibiotic, iv) minimal media with
118 ampicillin.
119
120 **Small Colony Variants Characterization**
121
122 To determine what these SCVs are, we isolated 6 independently generated SCVs of
123 MG1655 and characterized them phenotypically and genotypically. Firstly, to determine if
124 the SCVs are a form of resistance that has emerged over the long term experiment, we
125 determined their MIC to the drugs they were previously exposed to (Figure 3). Each SCV
126 showed at least a 5-fold change over the ancestral MG1655's MIC. Each SCV has a distinct
127 antibiotic sensitivity profile in terms of collateral sensitivities and cross-resistances
128 (Supplemental Figure 3).
129
130 Notably, although these SCVs are resistant to the bacteriostatic antibiotic to which they
131 were exposed, there is very little growth in the long term culture over the course of 30 days.
132 To determine why a marked increase in density does not occur despite the evolution of
133 resistance, we performed OD-based growth experiments of the SCVs with different
134 concentrations of antibiotics. We found that even though these mutants are resistant, their
135 growth rates and the maximum optical densities decreased proportionally to the drug
136 concentration and their lag time was substantially increased (Supplemental Figure 4). This
137 result is consistent with previous observations [15].
138
139 The small colonies obtained from both CHL and AZM cultures appear unstable, that is,
140 when streaked on LB plates without the drug both small colonies and normal sized colonies
141 appear. After the genotypic characterization of these SCVs we do find genetic differences
142 in most of them (Supplemental Table 1), but we could not find a clear mechanism that
143 would explain this resistance.
144



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146 **Fig 3. MIC of the SCVs to their respective drugs.** Three SCVs were isolated from each
147 condition from day 30 of the long term experiment, grown up in 1.5x *E. coli* MG1655's
148 MIC for these respective drug, and then E-tested.
149
150

151 **Heteroresistance**

152 Antibiotic heteroresistance (HR) is defined as, "a phenotype in which a bacterial isolate
153 contains subpopulations of cells that show a substantial reduction in antibiotic
154 susceptibility compared with the main population", and is detected via a population
155 analysis profile (PAP) test [16]. The revertant populations obtained from these SCVs have a
156 lower MIC than those of their small colony ancestor. These revertant populations are
157 capable of rapidly regenerating the SCVs, which have a higher MIC – meeting the
158 definition of antibiotic HR. The ancestral *E. coli* MG1655 is not capable of generating
159 resistant subpopulations, as shown via PAP test (Supplemental Figure 5 Panels A and B).
160 In Figure 4, we show PAP tests of a CHL SCV (Panel A) and an AZM SCV (Panel B).
161 Both SCVs are shown to be heteroresistant based on the above criteria. Moreover, highly
162 resistant colonies isolated from these PAP tests were found to be unstable and revert to the
163 initial SCV state in 15 days. As shown in Supplemental Figure 5 Panels C and D, all SCVs
164 obtained from the long term experiment meet the criteria for HR.
165
166
167
168

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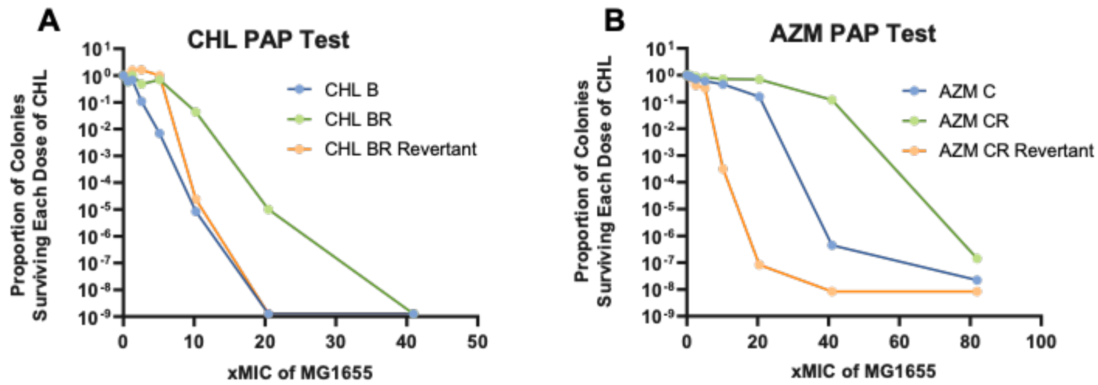
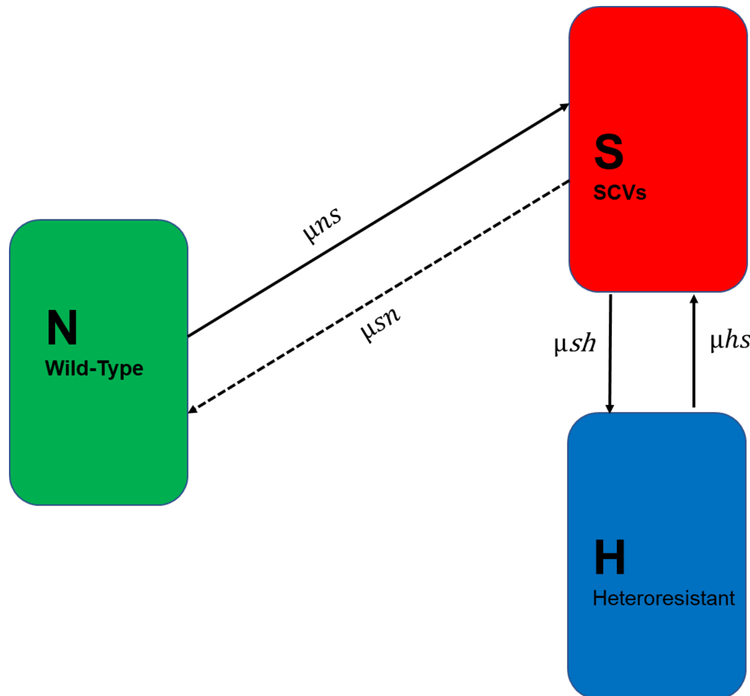


169
170 **Fig 4. PAP tests of a CHL and an AZM SCV. (A)** PAP tests of a CHL SCV (blue line),
171 the most resistant isolate of this clone (green line), and the most resistant isolate after being
172 grown without antibiotic pressure for 15 days (orange line). **(B)** PAP tests of an AZM SCV
173 (blue line), the most resistant isolate of this clone (green line), and the most resistant isolate
174 after being grown without antibiotic pressure for 15 days (orange line).
175
176 **Mathematical Model and Computer Simulations**
177
178 To explore the generality of our experimental results, we constructed a mathematical and
179 computer-simulation model. In Figure 5 we depict the model graphically and in the
180 Supplemental Text we describe the model and its equations.
181



182
183 **Fig 5. Diagram of a semi-stochastic model of the evolution of HR.** The variables N, S,
184 and H are, respectively, the wildtype *E. coli* MG1655, SCVs, and the heteroresistant
185 bacteria in cells/mL. The parameters μ_{ns}, μ_{sn}, μ_{sh} and μ_{hs} are the transition rates, per cell
186 per hour, between the different states.

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187

188 In Supplemental Figure 6A we illustrate how the presence of an antibiotic selects for the
189 emergence and ascent of resistant SCVs and a heteroresistant population from an initially
190 wild-type population. In Supplemental Figure 6B, we show that an initial population of
191 SCVs when grown without antibiotics will rapidly transition and give rise to a
192 heteroresistant population which ascends and becomes limited by the resource. In
193 Supplemental Figure 6C, we show that exposing the heteroresitant population to the drug
194 more rapidly selects for the emergence and dominance of reistant SCVs than when
195 exposing the wild type population to the same concentraton of the drug. In Supplemental
196 Figure 6D, we show the changes in average MIC for the three scenarios depicted in panels
197 A, B, and C.

198

199

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200 **Discussion**
201
202 The canonical distinction between bacteriostatic and bactericidal antibiotics has deeply
203 influenced their clinical usage. Traditionally, bacteriostatic antibiotics were considered
204 "weaker drugs", but this traditional view is questionable. Drugs which are classified as
205 bacteriostatic can and do kill bacteria in a concentration dependent manner [17]. Moreover,
206 meta-analysis studies do not demonstrate differences in the clinical success of therapy with
207 either types of drugs even in severe infections [11]. Indeed, bactericidal agents could be
208 reserved for life-threatening infections, particularly in immunocompromised patients or
209 those suffering from chronic infections. A more extended use of bacteriostatic drugs could
210 be beneficial to spare the use and overuse of bactericidal antibiotics which fosters
211 resistance. A limitation to progress in the extended use of bacteriostatic drugs is the
212 shortage of pharmacodynamic (PD) data with these drugs.
213
214 The distinction between bacteriostatic and bactericidal antibiotics is confounded by another
215 factor: The primary cellular and molecular targets do not necessarily differ between these
216 classes of drugs. Several bacteriostatic antibiotics have mechanisms of action that one
217 would anticipate being bactericidal: mecillinam and cefsulodin inhibit cell wall synthesis
218 [18]; novobiocin inhibits DNA gyrase [19]; and rifampin inhibits the DNA-dependent RNA
219 polymerase which is bacteriostatic for *E. coli* and bactericidal for *Mycobacterium* [20,21].
220 Most notably, drugs which target the ribosome and inhibit protein synthesis can be either
221 bactericidal (such as gentamicin or tobramycin) or bacteriostatic (such as the macrolides,
222 phenicols, tetracyclines, oxazolidinones, and spectinomycin) [22]. Interestingly, the potential
223 bactericidal effect of one of the ribosome-targeting drugs, chloramphenicol, is prevented
224 by the production of (p)ppGpp in the exposed cells [23]. This suggests that the difference
225 between a bacteriostatic drug and a bactericidal one is a property of the treated cell rather
226 than the antibiotic [24]. All the above examples illustrate the need for a better understanding
227 of the PD, population biology, and evolutionary biology of treatment with bacteriostatic
228 antibiotics.
229
230 In this study, we present evidence that bacteriostatic drugs of two different classes (the
231 phenicols and macrolides) inhibit the growth of *E. coli* for extended periods, i.e. 30 days,
232 and moreover maintain the culture in a kind of stationary phase where the density of viable
233 bacterial cells is stable. Although, unlike stationary phase we found there to be an
234 abundance of the limiting resource, implying that the cultures remain drug-limited even
235 after a month. Most interestingly, despite the fact that the bacteria in the population appears
236 to not be replicating due to the lack of net growth, evolution still occurred. A population
237 of small colony variants (SCVs) emerged and ascended to become the dominant population
238 of bacteria. We attribute this evolution to the fact that even though the population at large
239 was neither increasing nor decreasing, the population was replicating at a rate roughly
240 equal to that at which it was dying. This finding is unanticipated and inconsistent with the
241 common perception that bacteriostatic drugs simply arrest bacterial growth. This result
242 questions the definition of bacteriostasis.
243
244 These SCVs were found to be highly resistant not only to the challenging agents, but even
245 to some types of bactericidal agents, such as aminoglycosides and rifampin. Curiously, the

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246 resistance of the SCVs to chloramphenicol (CHL) and azithromycin (AZM) are not due to
247 the canonical resistance mechanisms [25,26]. SCVs have been implicated in treatment failure,
248 primarily in *Staphylococcus aureus*, but there are limited reports of SCVs being associated
249 with treatment failure in *E. coli* [27,28]. We have yet to determine the genetic and molecular
250 basis of the observed SCVs, but they appear to be distinct from the previously described
251 mechanisms [29-33]. Certain mutations observed here (Supplemental Table 1) might account
252 for the SCV phenotype. For instance, in the case of AZM-induced SCVs, missense variants
253 of the *citG* gene, encoding the 2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A
254 synthase, were consistently found. This mutation might alter members of the GntR family
255 of transcriptional regulators which could influence the DNA-binding properties of the
256 regulator resulting in repression or activation of transcription, or could directly impact ATP
257 synthesis [34], leading to the generation of the SCVs. Additionally, mutations in either *acrAB*
258 which interferes with AZM membrane transport [35] or mutations in the gene encoding the
259 50S ribosomal protein L22, a known mechanism of macrolide resistance [36], account for the
260 increase in the MIC to AZM but does not account for the emergence of the SCVs. This is
261 also true in the case of CHL-induced SCVs in which we found mutations in the gene
262 encoding the 50S ribosomal protein L4, a CHL binding site [37]. We found a mutation in a
263 CHL-induced SCV in the gene encoding the Tyrosine-tRNA ligase. This mutation could
264 account for the generation of SCVs, since inhibitors of this ligase strongly decrease
265 bacterial growth, but this mutation was only found in one of the six SCVs [38]. Most
266 interestingly, we were unable to find any SNPs in one of the isolated CHL SCVs.
267
268 Unexpectedly, the antibiotic resistance observed here is transient, as would be anticipated
269 for heteroresistance (HR), suggesting a high fitness cost of the mutations detected. In
270 support of this HR hypothesis we found that these SCVs meet all the criteria set forth for
271 HR: there are subpopulations present at a frequency greater than 10^{-7}, with an MIC higher
272 than 8x that of the main population, and reversion of the resistant subpopulation occurs in
273 short order [39]. To our knowledge, this is the first report of both the spontaneous evolution
274 of HR as well as HR to bacteriostatic drugs.
275
276
277
278

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279 **Materials and Methods**
280
281 Bacterial Strains. *E. coli* MG1655 was obtained from the Levin Lab bacterial collection [40].
282 pAM34 with the origin of replication under control of an IPTG promoter and an ampicillin
283 resistance cassette was obtained from Calin Guet from IST Austria [14].
284
285 Growth Media. LB (Lysogeny Broth) (244620) was obtained from BD. The DM (Davis
286 Minimal) minimal base without dextrose (15758-500G-F) was obtained from Sigma
287 Aldrich (7 g/L dipotassium phosphate, 2 g/L monopotassium phosphate, 0.5 g/L sodium
288 citrate, 0.1 g/L magnesium sulfate, 1 g/L ammonium sulfate). MHII plates were made from
289 MHII broth (212322) obtained from BD. Glucose (41095-5000) was obtained from Acros.
290 LB agar (244510) for plates was obtained from BD.
291
292 Growth Conditions. Unless otherwise stated, all experiments were conducted at 37°C with
293 shaking.
294
295 Sampling bacterial densities. The densities of bacteria were estimated by serial dilution in
296 0.85% saline and plating. The total density of bacteria was estimated on LB agar plates.
297
298 Antibiotics. Chloramphenicol (23660) was obtained from United States Pharmacopeia.
299 Azithromycin (3771) was obtained from TOCRIS. Ampicillin (A9518-25G) was obtained
300 from Sigma Aldrich. Isopropyl β-D-1-thiogalactopyranoside (IPTG; I56000-5.0) was
301 obtained from Research Products International. All E-tests were obtained from
302 bioMérieux.
303
304 Estimating Minimal Inhibitory Concentrations. Antibiotic MICs were estimated using E-
305 tests on MHII plates or via broth microdilution [41,42].
306
307 Long Term Experiments. Flasks containing 10 mL of DM with 1000 µg/mL of glucose and
308 an initial density of 10^5 CFU/mL cells were grown at 37ºC with shaking for 30 days. *E.*
309 *coli* MG1655 was grown with either no drug, 4x MIC of CHL or 3x MIC of AZM. Samples
310 were taken every 5 days and plated on LB agar plates.
311
312 Long Term Experiments with non-replicative plasmid. Flasks containing 10 mL of DM
313 with 1000 µg/mL of glucose and an initial density of 10^5 CFU/mL cells were grown at
314 37ºC with shaking for 30 days. *E. coli* MG1655 pAM34 was grown with either no drug,
315 4x MIC of CHL or 3x MIC of AZM. Samples were taken every 5 days and plated on LB
316 agar plates as well as 100 µg/mL Ampicillin and 0.5 mM IPTG LB agar plates.
317
318 Sequencing. Complete genomes were obtained with hybrid Illumina/Nanopore sequencing
319 by SeqCenter. Samples were extracted from single colonies using Zymo Quick-DNA
320 HMW MagBead Kit. Oxford Nanopore Sequencing library prep was performed with PCR-
321 free ligation library prep using ONT's V14 chemistry. Long read sequencing was performd
322 using R10.4.1 flowcells on a GridION with basecalling performed by Guppy in Super High
323 Accuracy mode. Illumina libraries were prepared and sequenced per SeqCenter's
324 standards. Quality control and adapter trimming was performed with bcl-convert and

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325 porechop for Illumina and ONT sequencing respectively. Hybrid assembly with Illumina
326 and ONT reads was performed with Unicycler [43]. Assembly statistics were recorded with
327 QUAST [44]. Assembly annotation was performed with Prokka [45].
328
329 Growth Rate Estimation. Exponential growth rates were estimated from changes in optical
330 density (OD600) in a Bioscreen C. For this, 24-hours stationary phase cultures were diluted
331 in LB or glucose-limited liquid media to an initial density of approximately 105 cells per
332 mL. Five replicas were made for each estimate by adding 300µl of the suspensions to the
333 wells of the Bioscreen plates. The plates were incubated at 37°C and shaken continuously.
334 Estimates of the OD (600nm) were made every five minutes for 24 hours in LB and 48
335 hours in glucose-limited medium. Normalization, replicate means and error, growth rate,
336 lag and maximum OD were found using a novel R Bioscreen C analysis tool accessible at
337 https://josheclf.shinyapps.io/bioscreen_app.
338
339 Residual Growth. After 30 days the cultures were centrifuged and filtered through a 0.22
340 µm filter. Strain resistant for CHL (Strain 1012 from the US Center for Disease Control's
341 MuGSI Isolate Bank which is *cmlA5* positive), AZM (Strain 1007 from the US Center for
342 Disease Control's MuGSI Isolate Bank which is *mph(A)* positive), or just MG1655 were
343 added to the supernatants and allowed to grow for 24h.
344
345

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346 **Acknowledgments**
347 We would like to thank Danielle Steed for her discussion of the clinical implications and
348 relevance of this work. We would also like to thank Jason Chen and LM Bradley for their
349 unwavering assistance and support. We would also like to thank the other members of the
350 Levin Lab. BRL would like to thank the US National Institute of General Medical Sciences
351 for their funding support via R35 GM 136407 and the US National Institute of Allergy and
352 Infectious Diseases for their funding support via U19 AI 158080-02.
353
354 **Data Availability Statement**
355 All the data generated are available in this manuscript and its supporting supplemental
356 material. Copies of the genomes sequence here have been made available under the NCBI
357 BioProject ID PRJNA1032893.

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