bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Title: Antibiotic tolerance, persistence, and resistance of the evolved minimal cell, *Mycoplasma mycoides* JCVI-Syn3B.

Authors: Tahmina Hossain[1], Heather S. Deter[2], Eliza J. Peters[1], and Nicholas C. Butzin[1]*

Affiliations:
[1]Department of Biology and Microbiology; South Dakota State University; Brookings, SD, 57006; USA.
[2]Department of Bioengineering; University of Illinois Urbana-Champaign; Urbana, IL, 61801; USA.
*Corresponding author. Email: *nicholas.butzin@gmail.com*

Summary
Antibiotic resistance is a growing problem, but bacteria can evade antibiotic treatment via tolerance and persistence. Antibiotic persisters are a small subpopulation of bacteria that tolerate antibiotics due to a physiologically dormant state. Hence, persistence is considered a major contributor to the evolution of antibiotic-resistant and relapsing infections. Here, we used the synthetically developed minimal cell *Mycoplasma mycoides* JCVI-Syn3B to examine essential mechanisms of antibiotic survival. The minimal cell contains only 473 genes, and most genes are essential. Its reduced complexity helps to reveal hidden phenomenon and fundamental biological principles can be explored because of less redundancy and feedback between systems compared to natural cells. We found that Syn3B evolves antibiotic resistance to different types of antibiotics expeditiously. The minimal cell also tolerates and persists against multiple antibiotics. It contains a few already identified persister-related genes, although lacking many systems previously linked to persistence (e.g. toxin-antitoxin systems, ribosome hibernation genes, etc.).

Keywords
Minimal cell, evolution, antibiotic tolerance, antibiotic persistence, antibiotic resistance

Introduction
The evolution of antibiotic resistance is a pressing public health concern in the 21st century; antibiotic resistance results from one or more genetic mutations that counteract an antibiotic (Van den Bergh et al., 2016). Resistance is regarded as the primary culprit of antibiotic treatment failure, but bacteria also employ less publicized strategies to evade antibiotic treatment, namely antibiotic tolerance and persistence (Fisher et al., 2017; Harms et al., 2016; Lewis, 2010). Persisters are a subpopulation of tolerant cells, which can sustain longer against antibiotic treatment in comparison to slow-growing dying cells by entering a metabolically repressed state (non-multiplying cells) (Cabral et al., 2018; Fisher et al., 2017). Most antibiotics are only effective against growing cells, and by not growing, the persister population can survive longer even without being genetically resistant. Here, we define persisters based on a kill curve and the original paper where persistence was proposed (Bigger, 1944) (our definition of persistence is explained in detail in the discussion). Two types of persisters may exist, triggered persisters (formed by environmental stimuli) and spontaneous persisters (generated stochastically) (Balaban et al., 2019; Balaban et al., 2004; Sulaiman and Lam, 2020; Uruén et al., 2021), although spontaneous persister formation is controversial and evidence is sparse (Keren et al., 2004; Kim and Wood, 2016; Orman and Brynildsen, 2013). What makes persisters medically

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

47 relevant is that they can revive and give rise to a new progeny after antibiotic treatment; the new
48 progeny can be genetically identical to the original susceptible kin, and this process plays a
49 pivotal role in recurring infections (Wilmaerts et al., 2019b) (Fig. 1). Furthermore, evolutionary
50 studies have determined that repeated exposure to antibiotics over many generations rapidly
51 increases tolerance leading to antibiotic resistance (Balaban and Liu, 2019; Cohen et al., 2013;
52 Fridman et al., 2014; Liu et al., 2020; Schumacher et al., 2015; Sulaiman and Lam, 2020; Van
53 den Bergh et al., 2016).
54

Fig. 1. Persisters survive antibiotic treatment, reestablish the population when antibiotics are removed, and increase the odds of gaining resistance. Presumably, viable but nonculturable cells (VBNCs) can do this too (although we did not study VBNCs in this work).

55
56 The precise molecular mechanisms underlying persistence are still debated (multiple
57 mechanisms are likely to exist), albeit several genes have been implicated (Wilmaerts et al.,
58 2019a; Wu et al., 2015). Toxin-antitoxin (TA) systems have been implicated in persistence and
59 were previously thought to be the key players for persistence (Lewis, 2010, 2012; Wang and
60 Wood, 2011). Particularly important is the HipAB TA system, because a toxin mutant, *hipA7,*
61 increases persistence by impeding cellular growth in the absence of its cognate antitoxin *hipB*
62 (Moyed and Bertrand, 1983). Subsequent studies also report that overexpression of other TA
63 systems' toxins increased persistence (Correia et al., 2006; Kim and Wood, 2010; Korch and
64 Hill, 2006). In contrast, new research found that the deletion of 10 TA systems (~22% of TA
65 system in the genome) in *E. coli* does not have an apparent effect on persistence both at the
66 population and single-cell levels (Goormaghtigh et al., 2018), though *E. coli* can have more than
67 45 TA systems (Horesh et al., 2018; Karp et al., 2014; Xie et al., 2018). Though this work
68 supports that TA systems are not controlling persistence, the remaining 35 TA systems (~78%)
69 could be controlling this phenomenon. Mauricio *et al.* studied persistence on Δ12TA *Salmonella*
70 *enterica* and demonstrated that TA systems are dispensable (Pontes and Groisman, 2019),
71 although this strain has 18 predicted TA systems based on the TAfinder tool (Xie et al., 2018).
72 Another knockout study on *Staphylococcus aureus* deleted three copies of type II TA system
73 from the Newman strain (Conlon et al., 2016). But further studies identified several type-I
74 (sprG1/sprF1, sprG2/sprF2, sprG4/sprF4) (Riffaud et al., 2019) and type-II (SavRS) (Wen et al.,
75 2018) TA system in HG003 and NCTC-8325 strains, respectively. These are the parental strains
76 of Newman, and these TA genes are also found in the Newman strain (Sassi et al., 2015).
77 Moreover, a recent study showed that antitoxin sprF1 mediates translation inhibition to promote
78 persister formation in *S. aureus* (Pinel-Marie et al., 2021). These findings raise many questions
79 about TA systems' implication in bacterial persistence (Goormaghtigh et al., 2018; Kim and
80 Wood, 2016; Pontes and Groisman, 2019; Tsilibaris et al., 2007). There are major limitations of
81 studying TA systems in native bacteria due to their high abundance in most bacterial genomes;
82 they often have redundant and overlapping functions and can have interdependencies within
83 network clusters (Harms et al., 2018; Ronneau and Helaine, 2019; Wang et al., 2013).
84 Additionally, TA systems respond to a variety of stresses (e.g. bacteriophage infection, oxidative

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

85 stress, etc.), which creates a hurdle to probe their connection with any phenomenon related to
86 stress response, namely antibiotic tolerance and persistence (Harms et al., 2018; Kang et al.,
87 2018; Ronneau and Helaine, 2019). TA systems are also involved in other mechanisms in the
88 cell that respond to stress, such as the stringent and SOS responses (Ronneau and Helaine, 2019),
89 virulence (De la Cruz et al., 2013), and the regulation of pathogen intracellular lifestyle in varied
90 host cell types (Lobato-Marquez et al., 2015). Furthermore, new types of TA systems may be yet
91 unidentified. These challenges could be resolved using a strain that lacks canonical TA systems.
92 But TA systems are naturally abundant, and large-scale knockouts are both error-prone and
93 labor-intensive. We took advantage of the recently developed minimal cell that does not encode
94 any sequences displaying homology to known TA systems and showed that it can still form
95 persisters.
96 Several other mechanisms have also been considered in persister research including SOS
97 response, oxidative stress response, etc. (Trastoy et al., 2018; Wilmaerts et al., 2019a; Wilmaerts
98 et al., 2019b). Two extensively studied phenomenon related to persistence are cellular ATP
99 levels and (p)ppGpp levels. The accumulation of (p)ppGpp (stress sensing alarmone) mediates
100 the stringent response, which controls a stress-related persistence mechanism (Harms et al.,
101 2016). (p)ppGpp regulates many networks (such as ribosome dimerization) that can cause cells
102 to go into dormancy (Gaca et al., 2015; Song and Wood, 2020; Wood and Song, 2020). Another
103 well-studied model, *ATP depletion increases persistence*, has drawn much attention in persister
104 research (Conlon et al., 2016). This finding is consistent with REF (Pu et al., 2019), which
105 demonstrated that lower ATP levels lead to protein aggregation and increased tolerance. This
106 result is also coherent with our recently published data, which established that interfering with
107 protein degradation by forming a proteolytic queue at ClpXP will increase tolerance levels
108 dramatically (Deter et al., 2019a). Transcriptomic analysis of queuing-tolerant population
109 showed upregulation of genes related to metabolism and energy (Deter et al., 2020b). However,
110 other studies reported that ppGpp and ATP reduction are not essential for persistence (Bhaskar et
111 al., 2018; Chowdhury et al., 2016; Pontes and Groisman, 2019). These contradictory studies are
112 common in persister research, and we hypothesize these inconsistencies are due to the
113 interconnection of gene networks surrounding persistence.
114 One goal of our study is to clarify mechanisms using a minimal, simpler system. Research
115 over the last several years has resulted in a lot of discussion concerning genes essential for
116 persistence (Pontes and Groisman, 2019; Ronneau and Helaine, 2019; Wilmaerts et al., 2019a;
117 Wilmaerts et al., 2019b). Since persistence and tolerance are present in phylogenetically
118 diversified bacterial species (Meylan et al., 2018; Wilmaerts et al., 2019b), it is feasible that an
119 underlying genetic mechanism is conserved in evolutionarily related microorganisms, and the
120 most likely candidates are essential genes or the disruption of crucial networks. In our recent
121 work, we demonstrate that antibiotic tolerance in *Escherichia coli* may result from a whole-cell
122 response and can occur through multiple pathways or networks, which may work simultaneously
123 and cooperatively to survive against antibiotics (Deter et al., 2020b).
124 Our strategy to study the underlying mechanisms of persistence was to use a bacterial species
125 that contains mainly essential genes and networks with reduced complexity and fewer networks.
126 The minimal system can reveal hidden phenomena (Glass et al., 2017) of antibiotic survival. For
127 example, genes and networks that have previously been identified can be eliminated as causal if
128 the genome lacks them. In contrast, genes present in Syn3B that were previously identified in
129 other organisms can become the focus of the work. The reduced complexity has its limits as
130 there are likely several methods microbes use to survive antibiotics, and not all methods will be

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

131 in a minimal system. With these limitations well-understood, we explored antibiotic tolerance,
132 persistence, and resistance in the minimal cell *Mycoplasma mycoides* JCVI-Syn3B (called
133 Syn3B throughout), a synthetic genetic background that contains the least number of genes and
134 smallest genome of any known free-living organism (Syn3B contains 473 genes and its genome
135 is ~531 Kbp long, while *E. coli* contains >4,000 genes and its genome is ~4,600 Kbp long). The
136 Syn3B genome was minimized from Syn1.0 (contains a chemically synthesized genome of *M.*
137 *mycoides* subspecies capri with some watermarks and vector sequences) by removing non-
138 essential genes (Gibson et al., 2010; Hutchison et al., 2016). Syn3B consists predominantly of
139 essential and a few non-essential (added for ease of genome manipulation) and quasi-essential
140 genes (required for robust growth) (Hutchison et al., 2016). This microbe was designed to have a
141 minimal number of genes and networks with the expectation that many of the first principles of
142 cellular life could be explored in the simplest biological systems (Glass et al., 2017). We show
143 that Syn3B populations contain both persister and tolerant cells, and its genome contains a few
144 previously identified persister-related genes. This work establishes that many systems previously
145 shown to be related to bacterial persistence, such as TA systems and ribosome dimerization, are
146 not essential for persistence in the minimal cell, and it demonstrates the effectiveness of using
147 the minimal cell to study antibiotic survival.

148 **Result**

149 **Whole-genome analysis of the evolved minimal cell.** Although the minimal genome (Syn3B)
150 was designed for ease of genetic manipulation, Syn3B grows slowly and to a low cell density.
151 We adjusted the original SP4 media (Tully et al., 1979) to address these limitations. The new
152 media, SP16, allows for faster cell growth and higher yields in liquid cultures. We noticed that
153 Syn3B growth was slightly better after every subculture. Thus, we did a cyclic subculture of
154 Syn3B in our optimized media by passing cells during logarithmic growth. After 26 passages, we
155 observed better growth and isolated a single colony named Syn3B P026 (Pass 26). This strain
156 has a shorter lag phase and an increased growth rate; the average doubling time of P026 is
157 approximately 2.5 hours compared to the ancestral Syn3B doubling time of ~6 hours under the
158 same conditions (Fig. S1.A-B). We performed a whole-genome analysis of Syn3B P026 to
159 examine the genetic basis of these changes. Most of the mutations (9 of 11) in P026 are
160 intergenic except one synonymous (*fakA*) and one non-synonymous (*dnaA*) (Table 1). *fakA* is a
161 fatty acid kinase, and there is less evidence to suggest a direct connection to substantial
162 alterations in bacterial growth. *dnaA* is a positive regulator of DNA replication initiation.
163 Considering that bacterial growth rate is dependent on the frequency of DNA replication and that
164 *dnaA* mutants have been known to result in over-replication (Skarstad and Boye, 1994), we
165 hypothesize that the mutation of *dnaA* in P026 could be responsible for the higher growth rate.
166 We also sequenced the *dnaA* gene from 5 colonies of a P026 subculture, and they had similar
167 growth rates as the P026 culture we initially sequenced. All 6 colonies have the same mutation in
168 the *dnaA* gene. These results suggest that the *dnaA* is the likely cause for the increased growth
169 rate of the evolved strain P026 compared to the parent strain, but we did not further pursue the
170 role of *dnaA* because it is not the main focus of this study.
171
172
173
174
175
176

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Table 1. Mutation of *Mycoplasma mycoides* JCVI-Syn3B, and Whole Genome Sequence (WGS) analysis identified the mutations (bold and underlined). All antibiotic-resistant mutant strains were named based on the number of passes (P) in a specific antibiotic (K: Ksg, S: Strep, C: Cip, SC: Strep-Cip). All antibiotic-resistant mutants were selected from two separate evolutionary lineages, and named L1 for lineage 1 or L2 for linage 2.

Syn3B Strains	Intergenic mutation[a]	Mutation positions[b]	Genotype change	Amino acid substitution	Gene
Parent*					
P026	9	547	**G**CA → **A**CA	Ala → Thr	*dnaA*+
		274928	TA**C** → TA**T**	Tyr → Tyr	*fakA*c+
Ksg^R					
PK07-L1	8	479174	**C**CT → **A**CT	Pro → Thr	*rpoC*+
		3322	G**G**A → G**A**A	Gly → Glu	*ksgA*+
PK07-L2	8	479174	**C**CT → **A**CT	Pro → Thr	*rpoC*+
		3322	G**G**A → G**A**A	Gly → Glu	*ksgA*+
Strep^R					
PS04-L1	4	101452	A**A**A → A**G**A	Lys → Arg	*rpsL*+
		33652	**G**GA → **A**GA	Gly → Arg	*CDS_6*+
PS04-L2	3	101452	A**A**A → A**G**A	Lys → Arg	*rpsL*+
		147803	A**G**C → A**T**C	Ser → Ile;	*rpsD*+
		33652	**G**GA → **A**GA	Gly → Arg	*CDS 6*+
Cip^R					
PC06-L1	4	7735	**G**AA → **A**AA	Glu→ Lys	*gyrA*+
		305613	**G**AT → **A**AT	Asp→ Asn	*parC*+
PC06-L2	4	7735	**G**AA → **A**AA	Glu→ Lys	*gyrA*+
		305613	**G**AT → **A**AT	Asp→ Asn	*parC*+
		304707	G**A**T → G**C**T	Asp→ Ala	*parE*+
		33652	**G**GA → **A**GA	Gly → Arg	*CDS_6*+
Strep^R-Cip^R					
PSC09-L1	4	7725	AG**T** → AG**G**	Ser→ Arg	*gyrA*+
		101452	A**A**A → A**G**A	Lys → Arg	*rpsL*+
		33652	**G**GA → **A**GA	Gly → Arg	*CDS_6*+
		305602	A**G**T → A**T**T	Ser → Ile	*parC*+
PSC09-L2	4	7725	AG**T** → AG**G**	Ser→ Arg	*gyrA*+
		101452	A**A**A → A**G**A	Lys → Arg	*rpsL*+
		33652	**G**GA → **A**GA	Gly → Arg	*CDS_6*+
		305602	A**G**T → A**T**T	Ser→ Ile	*parC*+

*Parent: High growth rate mutant parent to all of the strains in the table.
[a] Mutation in the non-coding DNA sequences located between genes.
[b] Genomic position numberings correspond to *Mycoplasma mycoides* JCVI-Syn3B and P026 (CP053944).
[c] Synonymous mutation: mutation does not change in the encoded amino acid sequence.
+ Mutated genes are likely to be functional, although the mutated genes' functionality has not been tested in this study.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

199 **Table 2.** Annotation of mutated genes from Table 1.

Mutated Gene	Annotation
dnaA[+]	Chromosomal replication initiator protein DnaA
fakA[+]	Dihydroxyacetone kinase
rpoC[+]	DNA-directed RNA polymerase subunit beta
ksgA[+]	16S rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))- dimethyltransferase
rpsL[+]	30S ribosomal protein S12
CDS_6[+]	Unknown
rpsD[+]	30S ribosomal protein S4
gyrA[+]	DNA gyrase subunit A
parC[+]	DNA topoisomerase 4 subunit A
parE[+]	DNA topoisomerase 4 subunit B

201 202 203 204 205 206 207 208 209 210

211 **Syn3B and evolved minimal cell P026 display antibiotic tolerance and persistence.** We
212 assessed tolerance and persistence (Fig. 2) of Syn3B (parent strain) and Syn3B P026 cultures
213 using two bactericidal antibiotics, ciprofloxacin (a fluoroquinolone) and streptomycin (an
214 aminoglycoside). Ciprofloxacin inhibits DNA replication by targeting DNA gyrase and
215 topoisomerase IV activity (Sanders, 1988). Streptomycin blocks protein synthesis by irreversibly
216 binding to the 16s rRNA of the 30S ribosomal subunit (Luzzatto et al., 1968). Syn3B and P026
217 were grown to stationary phase and diluted into fresh media containing the antibiotics to observe
218 population decay (see Methods). Both parent strain and P026 cultures showed a typical biphasic
219 death curve from stationary phase; the death rate became slower at ~20-24 h treatment with both
220 antibiotics compared to the earlier stage of population decay, which indicates Syn3B displays
221 persistence (Fig. 2. B-C). It appears that the survival was higher in the Syn3B ancestor strain
222 than P026 for both antibiotic treatments. We posit that it could be due to the slower growth rate
223 of ancestor strain, which is consistent with the literature, as several research groups already
224 establish that growth rate has a strong correlation with antibiotic susceptibility (Abshire and
225 Neidhardt, 1993; Lee et al., 2018; Pontes and Groisman, 2019; Tuomanen et al., 1986). As we
226 observed tolerance and persistence in both the ancestor strain and Syn3B P026, we decided to do
227 further analysis with Syn3B P026, because it was much easier to work.

228 Persister cells have been identified in native bacterial species in both stationary and
229 exponential phase cultures, and we tested if this was also true for the minimal cell. P026 was
230 grown to exponential phase and treated with antibiotics to determine whether the minimal
231 genome showed a similar biphasic death curve in exponential phase. As expected, we observed a
232 similar biphasic killing curve in exponential phase with slightly lower survival in exponential
233 phase compared to stationary phase for both antibiotics (Fig. 2. D). Moreover, we performed
234 resistance assays through the course of this work to rule out the possibility of resistance instead
235 of tolerance or persistence (see Methods), and no resistant colonies were identified. We then
236 tested if the surviving population had increased persister levels after antibiotic treatment. After
237 48 h of antibiotic treatment, the culture was passed into fresh media and then grown to stationary
238 phase. The culture was then exposed to the same antibiotic under the same condition, and as
239 expected, no significant difference between the two death curves was observed (Fig. S2).

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

240

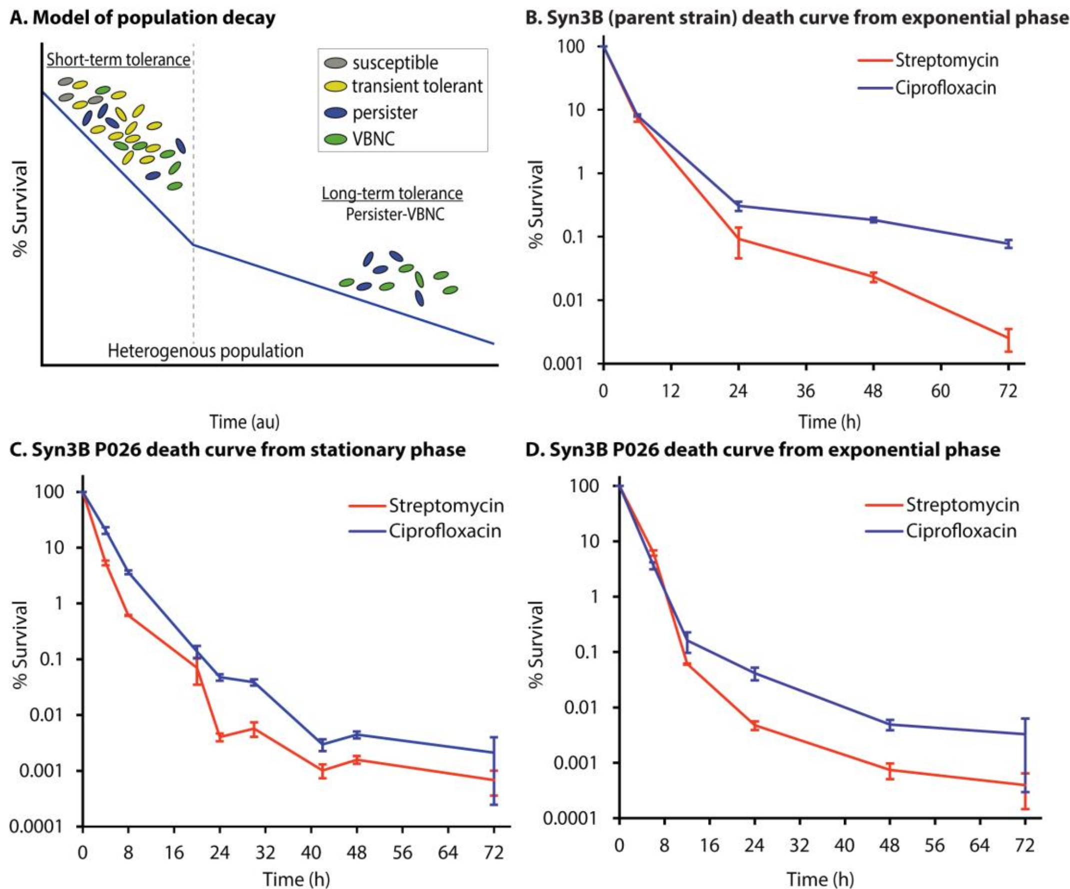


Fig. 2. Population decay during antibiotic treatment shows Syn3B persistence. A. A simplified model of population decay having two phases: short-term tolerance phase and long-term tolerance phase. Both phases contain heterogeneous populations. The short-term tolerant phase contains susceptible cells, slow-growing cells, transient tolerant cells, persister and viable but nonculturable cells (VBNCs). Transient tolerant cells survive longer than fast-growing susceptible cells, while transient tolerant cells die quicker than the persisters and VBNCs (VBNCs and persisters were not distinguished in this study). The long-term tolerant phase contains both persisters and VBNCs. **B.** Population decay of Syn3B (parent strain). Overnight cultures of Syn3B (parent strain) were grown to stationary phase, diluted to 1:10, and then treated with streptomycin (100 µg/mL) or ciprofloxacin (1 µg/mL) and sampled over time. Error bars represent SEM ($n \geq 3$). **C-D.** Population decay of Syn3B P026. Syn3B P026 cells were treated with streptomycin (100 µg/mL) or ciprofloxacin (1 µg/mL) and sampled over time. (**C**) Stationary phase cells were diluted 1/10 into fresh media containing antibiotics. Error bars represent SEM ($n \geq 6$). (**D**) Exponential phase cells were treated with streptomycin (100 µg/mL) or ciprofloxacin (1 µg/mL) and sampled over time. Error bars represent SEM ($n \geq 3$). There is 100% survival at time zero because percent survival is determined by the surviving CFU/ml compared to the CFU/ml at time zero.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

241 At this point, we have demonstrated that tolerance and persistence can be detected in Syn3B
242 cultures during both stationary and exponential phase, and this is consistent with native bacterial
243 species. Another well-known phenotype of antibiotic survival is that the tolerant population
244 increases with co-treatment of bacteriostatic and bactericidal antibiotics (Lewin and Smith, 1988)
245 (pre-treatment is another method (Kwan et al., 2013)). To use the minimal cell as a model of
246 antibiotic survival, this phenomenon should also be observed. Bacteriostatic antibiotics can
247 counteract the bactericidal antibiotic's killing activity by arresting the growth of rapidly growing
248 cells, which increases tolerance to the antibiotics (Kwan et al., 2013). Chloramphenicol is a
249 bacteriostatic antibiotic that inhibits translation by binding to bacterial ribosomes and inhibiting
250 protein synthesis, thereby inhibiting bacterial growth (Volkov et al., 2019). We co-treated with
251 the bacteriostatic antibiotic chloramphenicol and either streptomycin or ciprofloxacin. As
252 expected, the overall percent survival with chloramphenicol co-treatment increased compared to
253 streptomycin or ciprofloxacin alone after 24 h and 48 h (Fig. 3). These results support that
254 inhibition of translation by co-treating the cell with chloramphenicol increases tolerance in P026.
255

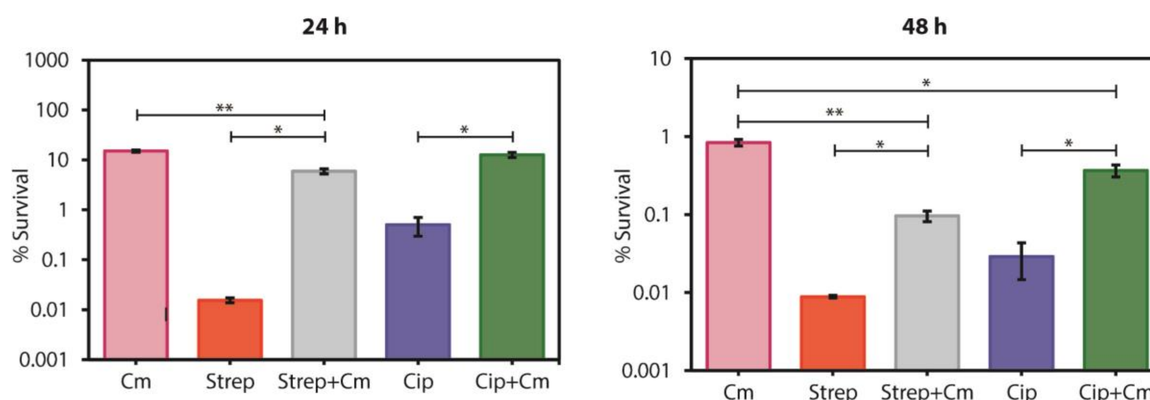


Fig. 3. The co-treatment of bactericidal antibiotic (streptomycin (Strep) or ciprofloxacin (Cip) with a bacteriostatic antibiotic (chloramphenicol (Cm)) shows increased survival of cells for 24 h (left) and 48 h (right) in Syn3B P026. Error bars represent SEM (n ≥ 3). $*p<0.05$. $**p<0.01$.
256
257 **The minimal genome contains previously identified genes related to tolerance and**
258 **persistence.** The mechanisms of persister formation are complex, but recent studies identify
259 several genes involved in this process (Cameron et al., 2018; Wilmaerts et al., 2019a). A few of
260 these genes are present in the minimal genome. We identified known and predicted genes related
261 to tolerance and persistence in the Syn3B genome (Table 2). During nutrient limitation, bacteria
262 generally switch their gene expression profile from rapid growth to a survival state by (p)ppGpp
263 levels (a hallmark of the stringent response), which is regulated by the RelA protein in *E. coli*
264 (Boutte and Crosson, 2013). Another important gene in the stringent response is *phoU*, a global
265 negative regulator, which is deactivated upon inorganic phosphate (Pi) starvation. Mutation or
266 deletion of this gene in other microorganisms leads to a metabolically hyperactive state and
267 decreased persistence (Li and Zhang, 2007; Zhang and Li, 2010). The SOS response is another
268 signaling pathway that upregulates DNA repair functions, which appears to be linked to bacterial
269 persistence. Genes related to the SOS response have been found upregulated in *E. coli* persister
270 (e.g. *uvrA, uvrB*) (Keren et al., 2004), and other mutants (such as *xseB*) have lost their ability to
271 induce detectable levels of persistence when pretreated with rifampicin, a bacteriostatic antibiotic
272 (Cui et al., 2018).

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

273 Other literature advocates the connection of persistence with energy metabolism, although the
274 outcomes are often inconsistent between models (Wilmaerts et al., 2019a). For example, the
275 deletion of *atpA* (encoding for ATP synthase subunit alpha) decreases the persister fraction
276 (Lobritz et al., 2015), but deletion of genes encoding other parts of the ATP synthase (*atpC and*
277 *atpF*) increases the persister fraction (Girgis et al., 2012; Kiss, 2000). Several heat shock
278 proteins, mainly proteases (e.g. *lon* (Pu et al., 2016; Wu et al., 2015)) and chaperons (e.g. *dnaK*
279 (Wu et al., 2015)*, dnaJ* (Hansen et al., 2008)*, clpB* (Wu et al., 2015)), are related to persistence
280 considering that deletion of those genes decreases persistence.
281 Another survival mechanism connected to persistence is trans-translation, which allows
282 bacteria to recycle stalled ribosomes and tag unfinished polypeptides for degradation, which in
283 *E. coli* requires tmRNA (encoded by *ssrA*) and a small protein (smpB). The deletion of these
284 genes causes persister-reduction in *E.coli* (Liu et al., 2017; Wu et al., 2015; Yamasaki et al.,
285 2020). Additional genes (*glyA* (Cui et al., 2018)*, galU* (Girgis et al., 2012), *trmE* (Cui et al.,
286 2018), *efp* (Cui et al., 2018)*, ybeY* (Cui et al., 2018), etc.; see Table 2) are indirectly related to
287 stress response or metabolism and have been reported to affect persistence. Overall, this shortlist
288 (Table 2 compare to Table S1) of genes demonstrates that there are far fewer genes to explore in
289 the minimal cell than any other known free-living microorganism on the planet. However,
290 further exploration is needed to test if there is a relationship between genes in Table 2 to
291 antibiotic survival in the minimal cell.
292

Table 3. Syn3B contains genes previously shown to be related to *E.coli* persistence and tolerance.

Gene	Protein/RNA	Major function	REF
dnaK, dnaJ, clpB	Chaperon	Global regulator	(Hansen et al., 2008; Wu et al., 2015)
phoU	Phosphate-specific transport system accessory protein	Global regulator	*(Li and Zhang, 2007)*
xseB	Exodeoxyribonuclease	DNA mismatch repair and recombination	(Cui et al., 2018)
uvrA, uvrB	Endonuclease	SOS response	(Cui et al., 2018; Keren et al., 2004; Wu et al., 2015)
relA	GTP pyrophosphokinase	Stringent response	(Wu et al., 2015)
lon	Protease	Protease	(Wu et al., 2015)
glyA	Serine hydroxymethyltransferase	Metabolism	(Cui et al., 2018)
atpA, atpC, atpF	ATP synthase	ATP synthesis	(Girgis et al., 2012; Kiss, 2000; Lobritz et al., 2015)
galU	UTP--glucose-1-phosphate uridylyltransferase	Lipopolysaccharide precursor synthesis	(Girgis et al., 2012)
trmE; efp; ybeY	tRNA modification GTPase; Elongation factor P; Endoribonuclease	Translation	(Cui et al., 2018)
ssrA; smpB	Transfer messenger RNA (tmRNA); Small Protein B	Trans-translation	(Wu et al., 2015; Yamasaki et al., 2020)

293
294

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Evolution of the minimal cell against different types of antibiotic and whole-genome analysis of the resistant strain. Up until this point, we have focused on antibiotic tolerance and persistence. For the minimal system to be also useful as a model of antibiotic resistance, it must be able to evolve resistance without horizontal gene transfer (i.e. isolated from other organisms). We hypothesize that due to lack of complexity and less control of mutation, simpler cells likely evolve faster than more complex organisms; a simpler, slimmed-down genome allows for rapid evolution to selective pressures. We selected different classes of bactericidal antibiotics including streptomycin (Strep), ciprofloxacin (Cip), a combination of streptomycin and ciprofloxacin (Strep-Cip), and one bacteriostatic antibiotic-kasugamycin (Ksg), to study the evolution of antibiotic resistance in the minimal genome. We applied cyclic antibiotic treatments where we repeatedly regrew and retreated the culture with the lethal dose of the same antibiotic for a few cycles with two biological replicates (Fig. S3B.) to make resistant mutants. In seven passes or less (nine passes with two antibiotics), the minimal genome rapidly evolved resistance to all antibiotics tested. We isolated single colonies (two separate evolutionary lineages named L1 for lineage 1 or L2 for linage 2) from each resistant mutants named Syn3B PS04 (4 Passes in Streptomycin), PC06 (6 Passes in Ciprofloxacin), PSC09 (9 Passes in Streptomycin and Ciprofloxacin), and PK07 (7 Passes in Kasugamycin). We then analyzed the whole genome for mutations. Most mutations were in intergenic regions of the resistant mutants, similar to Syn3B P026 (Table 1 and 2).

We observed Syn3B P026 quickly evolved against streptomycin, as it only took 4 passes to become resistant. Only two non-synonymous mutations were found in both lineages. The first one is in the *rpsL* gene (encoding the S12 ribosomal protein), which is frequently identified in streptomycin-resistant strains (Villellas et al., 2013), and the other one is the *CDS_6* gene, the function of which is not yet known. In PS04_L2, we also observed another mutation in the *rpsD* gene (encoding the S4 ribosomal protein). We found that Syn3B P026 took 6 and 9 cycles to gain resistance against ciprofloxacin and a combination of both streptomycin and ciprofloxacin, respectively. In both replicates of PC06, we observed mutations in *gyrA* (encoding the DNA gyrase subunit A) and *parC* (encoding DNA topoisomerase 4 subunit A). In PC06_L2, we observed another two mutations, one in *parE* (encoding DNA topoisomerase 4 subunit B) and another in the *CDS_6* gene. For the combined streptomycin-ciprofloxacin resistant strains, both lineages showed similar mutations in *rpsL, gyrA*, *parC*, and *CDS_6* genes. In the kasugamycin-resistant strain PK07, only two nonsynonymous mutations were found. The first one is in the *ksgA* gene, which encodes a methylase that modifies 16S rRNA, and inhibition of this protein by the antibiotic kasugamycin causes susceptibility. Therefore, when *ksgA* is inactivated, cells became kasugamycin-resistant (Mariscal et al., 2018; van Gemen et al., 1987). We reasoned that the nonsynonymous mutation in *ksgA* in PK07 makes the protein inactive and able to confer kasugamycin resistance. The other detected non-synonymous mutation is in *rpoC* (RNA polymerase subunit).

Discussion

Overall, we have demonstrated that the minimal cell contains both antibiotic tolerant and persistent subpopulations, and it can quickly evolve to gain resistance. We have successfully generated an evolved strain of the minimal cell Syn3B P026, which has a better growth rate than the ancestral strain and overcomes one of the major difficulties of working with the minimal cell. We show that Syn3B can be used as a model for studying antibiotic survival, especially when we consider that the minimal genome's core proteins are present in many other microorganisms

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

341 including human pathogens. Syn3B has over 50% similarity to human pathogens that have been
342 identified as a concern with respect to the growing antibiotic-resistance problem (Table S2)
343 (Centers for Disease Control and Preventions, 2019). For example, out of 455 protein-encoding
344 genes of Syn3B, 338 of them (74%) have homologs to *S. aureus* NCTC 8325 proteins. *S. aureus*
345 causes meningitis and pneumonia, and antibiotic resistance is a major problem of this organism
346 (Foster, 2017).
347 The definition of persistence is often debated, but we argue that the original definition
348 proposed by Biggers in his seminal 1944 paper (Bigger, 1944), where persisters were first
349 classified and named, is sufficient. Syn3B meets 9 out of 10 characteristics defined by Bigger.
350 The only characteristic not found in Syn3B (point 4 in Table 4) was an untested hypothesis that
351 Bigger put forward about persisters, which states that persisters can be induced without antibiotic
352 stress (now known as spontaneous persisters). This characteristic has yet to be resolved and was
353 not addressed in our work. Bigger made it clear that persister identification should occur over
354 several days and suggested 72 h (Bigger, 1944). We tested Syn3B for 72 h (Fig. 2B-D), and it
355 formed persisters. Though we agree with Bigger's original assertion that prolonged antibiotic
356 treatment is required to demonstrate persistence, the originally 72 h cutoff does not consider
357 different dividing times of bacteria, which is likely to affect killing rates.
358 A more recent definition of tolerance and persistence was put forth by a consensus statement
359 released after a discussion panel with 121 researchers (Balaban et al., 2019). They defined
360 persistence similarly to the original Bigger's paper with some slight changes. They defined
361 persistence as a subpopulation of tolerance, and this is generally agreed upon in the literature.
362 They then stated that the "tolerance state" could be distinguished from the "persistence state"
363 based on a killing curve. We agree that persisters can be distinguished from other populations
364 using a kill curve (Fig. 2A), the Bigger paper also demonstrated the importance of prolonged
365 antibiotic treatment to identify persisters (Bigger, 1944), and we showed that Syn3B also has a
366 distinguished death curve (Fig. 2B-D). However, we do not call the first death phase "tolerance,"
367 as proposed by REF (Balaban et al., 2019), because naming this subpopulation tolerance is easily
368 confused when the entire population is tolerant. We, instead, label the first phase (faster death
369 rate) of the death curve as short-term tolerance phase contains heterogeneous population includes
370 susceptible cells, transient tolerant cells (caused by slow-growth or heterogeneity in gene
371 expression (El Meouche and Dunlop, 2018; Lee et al., 2019)), persisters and VBNCs.
372 Susceptible cells are included because it will take time to kill off these cells regardless of the
373 antibiotic used. Transient tolerant cells can tolerate antibiotics longer than susceptible cells, but
374 not as long as VBNCs or persisters (VBNCs were not investigated in this study). We termed the
375 second phase (slower death rate) as a long-term tolerance phase containing persister and VBNCs.
376 We described in our definition that a death curve can distinguish short-term tolerance and
377 persistence.
378 Few researchers have put forth that the definition for persisters should include a rapid decline
379 in the short-term tolerance stage and then a persister plateau (Bartell et al., 2020; Mulcahy et al.,
380 2010; Sahukhal et al., 2017; Song and Wood, 2021). Depiction of persisters as a plateau may
381 underrepresent the heterogeneity of the population (Kaldalu et al., 2016), and a true plateau have
382 a slope of zero for an extended period of time, which we do not see in our Syn3B and *E.coli* data.
383 We first tested if a plateau is present in the model organism *E. coli*. Since other researchers (Aedo
384 et al., 2019; Mohiuddin et al., 2020; Song et al., 2019) and ourselves (Deter et al., 2020a) have
385 shown that *E. coli* forms persisters after three hours of ampicillin treatment at bactericidal
386 concentrations (100 μg/mL treatment), we reanalyzed our previously published data (Deter et al.,

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

387 2020a) and included 24 ampicillin exposure data to check for a plateau using a simple t-test point
388 comparison. The hypothesis is there is no plateau, the slope of the line is not zero, and a slow
389 population decay after 3 h. The null hypothesis is there is a plateau, the slope of the line is zero,
390 and the population reaches a steady-state with no decay after 3 h. Comparing the p-values of 3 h
391 ampicillin exposure to later exposure times could test if the population is a plateau (no significant
392 difference) or a slow decline (significant difference). As we suspected, the long-term *E. coli* death
393 curve slope is not zero, the cells are not in a plateau, and cells are dying slowly. This is evident by
394 the fact that 3 h ampicillin treatment is significantly different ($p<0.05$) than the that longer
395 exposures of ampicillin treatment: the p-values for 6 h, 8 h, and 24 h, were 6.5E-05, 4.6E-04, and
396 3.3E-06, respectively, compared to the 3 h ($n\geq3$) (Table S4). There are points in the death curve
397 where small plateaus ($p>0.5$) can be observed but overall, no plateau. This work underscores the
398 importance of doing long-term kill curves as laid out in REF (Balaban et al., 2019). Next, we tested
399 Syn3B P026 persisters and as expected, there was no plateau, the slope is not zero for different
400 antibiotics (Fig. 2C), and there is a slow decline in persistence over time. This is evident by the
401 fact that 24 h ciprofloxacin treatment was significantly different ($p<0.05$) than longer exposures
402 of ciprofloxacin treatment: the p-values for 48 h and 72 h were 5.2E-05 and 3.6E-02 compared to
403 the 24 h ($n\geq3$). Similar results were obtained for streptomycin (100 µg/mL); the p-value for 48 h
404 and 72 h treatment compared to 24 h is 4.2E-3 and 3.3E-2 ($n\geq3$). Similar to *E. coli,* there were
405 some points in the death curve that formed small plateaus ($p>0.5$), which again underscores the
406 importance of doing long-term kill curves. Our results support a rapid decline in the short-term
407 tolerance stage and then a slow decline in the persister stage, not a plateau, which aligns with the
408 historical data (Bigger, 1944) and modern definition (Balaban et al., 2019).
409 After establishing that Syn3B forms persisters, we looked into genes previously shown to be
410 related to tolerance in other organisms. We also find that the Syn3B genome lacks homologs of
411 several genes and pathways reported in earlier research to be related to tolerance and persistence
412 (Table S1). For instance, TA systems are often implicated for persistence and are still referenced
413 as causing persistence (Moreno-del Álamo et al., 2020; Riffaud et al., 2020; Shenkutie et al.,
414 2020), although recent research provided evidence of a lack of causation between persister
415 formation and TA system (Conlon et al., 2016; Pontes and Groisman, 2019). In our study, we
416 identify no known TA systems or homologous genes based on the results from TADB 2.0 (a
417 database designed to search for TA systems based on homology) (Xie et al., 2018) in Syn3B.
418 Our study clearly shows that TA systems are not required for tolerance or persistence in the
419 minimal cell, and it seems likely that most bacteria do not require them either. It is not surprising
420 that the minimal genome does not contain TA systems because the genome was designed by
421 researchers and not subject to the natural environment. Natural TA systems are often described
422 as "addictive" systems that are hard to lose once acquired; they often have overlapping toxin and
423 antitoxin genes, making mutations less likely to be selected. Curiously, we observed some
424 overlapping genes that are not at all similar to TA systems (they are also much longer in
425 sequence than traditional TA systems, and are not homologous to any known TA system), and
426 whose functions are not yet defined (Table S3).
427 Recently, the Wood group (Song and Wood, 2020; Wood and Song, 2020) proposed the
428 ppGpp ribosome dimerization persister (PRDP) model for entering and exiting the persister state
429 where ppGpp induces *hpf, rmf*, and *raiA*, which converts active ribosomes into their inactive
430 state (such as 100s ribosome) to reduce translation, and consequently cells enter into persistence.
431 Upon removal of antibiotic stress, cAMP level is reduced and HflX production is stimulated,
432 which dissociates inactive 100S ribosomes into active 70S ribosomes and growth resumes.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

433 However, we did not detect homologs to the required genes, *raiA, rmf, hpf, and hflX*, for
434 ribosome dimerization in the minimal cell genome, which means ribosome dimerization is not an
435 essential mechanism for this organism (or another unknown mechanism for ribosome
436 dimerization exists in Syn3B). (p)ppGpp may still play a role in Syn3B persistence, the genome
437 contains *relA* (converts ATP and GTP to (p)ppGpp; Table 3). A potentially fruitful study would
438 be to study the effects on survival by altering (p)ppGpp of Syn3B cultures.
439 This strain shows that TA systems and ribosome hibernation genes are not required for
440 bacterial tolerance or persistence. Moreover, there are far fewer genes (less than 20 genes)
441 present in Syn3B, which have been shown related to persistence. Thus, if specific genes or
442 regulons are responsible for persistence, then Syn3B should be very useful in identifying them in
443 future studies because it has a minimal number of genes. It is possible that persister formation
444 and maintenance results from slowed or disrupted cellular networks (this is the hypothesis we
445 most prescribe to), rather than the activity of specific genes or regulons. If tolerance and
446 persistence results from slowed or disrupted cellular networks, then Syn3B is an ideal model
447 organism to use because it has a minimal number of networks. While different genes and
448 networks are likely responsible for persistence depending on the antibiotic, strain, or bacterial
449 species, identifying genes in this minimal system should be applicable to a number of other
450 microorganisms including the pathogen *Mycoplasma mycoides* from which Syn3B was derived.
451 Regardless of how cells enter the dormant state, Syn3B provides a new model to study the genes
452 and networks that allow cells to survive antibiotic treatment and could pave the way for finding
453 new drugs that target the persister and tolerant subpopulations.
454
455
456
457
458
459
460
461
462
463
464
465
466
467
468
469
470
471
472
473
474
475
476
477
478

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

479 **Table 4.** In Bigger's 1944 paper, he identified cells that survived antibiotics longer, named them
480 persisters, and listed 10 characteristics of the persister subpopulation (Bigger, 1944). Syn3B
481 meets 9 out of 10 characteristics defined by Bigger. Left column: summary of Bigger's
482 definition. Right column: similar characteristics of Syn3B persisters. We provided no evidence
483 related to point 4 but observed a similar phenotype as Bigger to support point 10.
484

	Bigger, 1944 persister definition	Syn3B persisters
1.	An antibiotic failed to kill a small population of bacteria.	Multiple antibiotics failed to kill a small population of Syn3B.
2.	Persisters are a small population initially present in the population.	Syn3B contains a small persister subpopulation among the susceptible population.
3.	Persister can be induced or changed based on the environment.	The persistence (surviving population) level is different when the culture originated from a stationary phase or exponential phase. The persistence level is different when cultures were co-treated with bacteriostatic and bactericidal antibiotics compared to the bactericidal antibiotic only; chloramphenicol and streptomycin, and chloramphenicol and ciprofloxacin, compared to the antibiotics alone.
4.*	*Untested hypothesis:* No evidence showed that persisters were produced by the presence of an antibiotic.	This was not tested for Syn3B persisters.
5.	Bacteriocidal concentrations of an antibiotic killed the susceptible population, but not persisters.	The Syn3B susceptible population was killed by bacteriocidal concentrations of more than one antibiotic type, but persisters were not.
6.	Persisters are likely insensitive to an antibiotic because they are dormant and non-dividing. The antibiotic used, penicillin, kills bacteria only when they divide.	Syn3B persisters also appear to be dormant and likely non-dividing, because the antibiotics used are more effective against dividing cells.
7.	Descendants of persisters are no more resistant to an antibiotic than the original population.	Syn3B descendants of persisters are no more resistant to an antibiotic than the original population.
8.	When the antibiotic is destroyed, the majority of persisters will emerge and grow normally.	Syn3B persisters grew once the antibiotic was removed or diluted below the MIC.
9.	The antibiotic prolongs the dormant phase but not indefinitely.	Syn3B persisters continue to die, but slowly, the longer they are exposed to antibiotics. There is no plateau but a slow decline in cell death.
10.*	*Untested hypothesis:* Persisters may be coming out of the dormant state, attempting to divide and being killed by the antibiotic.	This was not tested for Syn3B persisters. Syn3B persisters continue to die, but slowly, the longer they are exposed to antibiotics. There is no plateau but a slow decline in cell death.

485 * Points 4 and 10 were untested hypotheses of Bigger and were not tested in our work.
486

487 Limitations of the study

488 We observed that despite controlling methodology to the greatest extent possible, persister
489 levels varied considerably (far more than observed in our previous work with *E. coli*) (Deter et
490 al., 2019a) between experiments for both streptomycin and ciprofloxacin treatments (Table S5).
491 We hypothesize that this variability might be due to the stochastic fluctuations (noise) in gene
492 expression levels, which results in protein level variations even among genetically identical cells
493 in a similar environment (Soltani et al., 2016). We expect that gene expression and protein
494 production to be more erratic in Syn3B compared to natural microorganisms because this cell has

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

495 a designed genome lacking many control mechanisms and did not evolve to achieve some level
496 of internal cellular "equilibrium" like native cells have.
497 We did not test for other subpopulations that have been identified during antibiotic treatment.
498 We did not test for Syn3B transient tolerant cells, VBNCs, or spontaneous persisters.
499

500 **Methods**

501 **Microbial strains and media.** *Mycoplasma mycoides* JCVI-Syn3B (Hutchison et al., 2016) and
502 its derivatives were used in this study. Syn3B was a gift from Dr. John I. Glass from J. Craig
503 Venter Institute, La Jolla, CA, USA. For evolution and antibiotic survival assays, cells were
504 cultured at 37°C in SP16 media (57.5% 2X P1, 10.0% P4, 17.0% FBS, tetracycline 0.4% and
505 vitamin B1 0.5%) (see Table S6 for details), which was developed based on SP4 media (Tully et
506 al., 1979). All cultures were plated in SP4 agarose media (0.55% agarose) for colony counts.
507 Note that agar is not used because it inhibits growth.
508

509 **Evolution by serial passage.** Syn3B cultures were grown in 3 mL tubes at 37°C overnight in
510 SP16 media. Overnight exponential cultures were serially passaged after each cycle of growth by
511 transferring 30 µL of culture into 3 mL fresh media to initiate the next cycle of growth, and the
512 cycles continued until a satisfactory growth rate was observed (Fig. S3. A). The culture was
513 plated after 26 passages and a single colony was isolated, P026.
514

515 **Genome extraction, whole-genome sequencing, and identification of mutations.** For whole-
516 genome sequencing (WGS), a single colony of the evolved strains Syn3B P026 and all the
517 resistant mutants (PK07_L1, PK07_L2, PS04_L1, PS04_L2, PC06_L1, PC06_L2, PSC09_L1,
518 PSC09_L2) were isolated and inoculated into SP16 media for 24 h at 37°C. Next, genomic DNA
519 was harvested and purified using Genomic DNA Purification Kit (ThermoFisher) in accordance
520 with the manufacturer's instructions. For quality checks, DNA purity and concentration were
521 assessed by gel electrophoresis and Qubit Fluorimeter prior to sending for sequencing. Novogene
522 Ltd. sequenced the genomes using paired-end Illumina sequencing at 2 × 150 bp read length and
523 350 bp insert size. A total amount of ~1 µg of DNA per sample was used as input material for the
524 DNA sample preparation. Sequencing libraries were generated from the qualified DNA samples
525 using the NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the
526 manufacturer's protocol. For data processing, the original sequence data were transformed into
527 raw sequenced reads by CASAVA base calling and stored in FASTQ (fq) format. For subsequent
528 analysis, the raw data were filtered off the reads containing adapter and low-quality reads to
529 obtain clean data. The resequencing analysis was based on reads mapping to reference genome
530 of Syn3B by BWA software (Li and Durbin, 2009). SAMTOOLS (Li et al., 2009) was used to
531 detect single nucleotide polymorphism (SNP) and InDels.
532 We also used Oxford Nanopore Technologies (ONT) MinION long-read sequencer to search
533 for large insertions or gene duplication in all the evolved strains. ONT libraries were prepared
534 using *Ligation kit* (SQK-LSK109) according to the manufacturer's instructions. R 9.5 flow cell
535 (FLO-MIN107, ONT) was used for sequencing based on manufacture protocol. The flow cell
536 was mounted on a MinION Mk 1B device (ONT) for sequencing with the MinKNOW versions
537 19.12.5_Sequencing_Run_FLO-MIN107_SQK-LSK109 script. Then, reads were mapped
538 against reference genome Syn3B using Geneious Prime software version 2020.1.
539 (*https://www.geneious.com*). No large insertions were found in the sequenced genomes.
540

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

541 **Minimum inhibitory concentration (MIC) tests.** Overnight cultures were serially diluted and
542 plated onto SP4 agarose plates containing different concentrations of antibiotics (Strep, Cip, Cm,
543 and Ksg) to determine the MIC of each antibiotic. Plates were incubated 4-5 days at 37°C before
544 colony counts. MIC values were defined in this study as the lowest antibiotic concentration that
545 inhibit the growth of Syn3B (See Fig. S4).
546
547 **Antibiotic survival assays.** The schematic of the antibiotic survival assay from a stationary
548 phase culture is shown in Fig. S3. C. Briefly, an overnight culture was diluted 1:100 into pre-
549 warmed media and grown to exponential phase (OD_{600} 0.1-0.3). Next, the culture was separated
550 into three flasks (for three biological replicates) and grown at 37˚C until it reached stationary
551 phase (OD 0.45-0.55, which takes ~3-6 h). After that, each culture was diluted 1:10 into 100
552 µg/mL streptomycin (10X MIC) or 1 µg/mL (10X MIC) ciprofloxacin containing pre-warmed
553 media and kept at 37˚C shaking at 250 rpm for 72 h. Samples were taken at different time points
554 until 72 h for the time-kill assays. To remove the antibiotic before plating, 100 µl of each sample
555 was washed with 1.9 mL ice-cold SP16 media and collected by centrifugation (16,000 rpm for 3
556 min at 4˚C). Cells were then resuspended and serially diluted into ice-cold SP16 media and
557 plated to count the colony-forming units (CFU). Persisters were quantified by comparing CFUs
558 per milliliter (CFU/ml) before antibiotic treatment to CFU/ml after antibiotic treatment. Plates
559 were incubated at 37ºC for 4-5 days, then scanned using a flatbed scanner (Datla et al., 2017;
560 Deter et al., 2019a; Levin-Reisman et al., 2017). Custom scripts were used to identify and count
561 bacterial colonies (Deter et al., 2019a; Deter et al., 2019b) used to calculate CFU/ml and
562 persister frequency. Over 200 colonies were streaked periodically into antibiotic-containing
563 plates to test for antibiotic-resistant mutants. Also, antibiotic-treated culture was washed after 48
564 h, regrew to stationary phase, and exposed the culture again in the same antibiotic treatment for
565 48 h and plated after 24 h and 48 h to observe the difference between the first (Strep/Cip) and
566 second death curve (Strep re-exposed/Cip re-exposed) in both antibiotic treatment. Persister
567 assay for *E.coli* 24 h ampicillin treatment (unpublished data) was done by similar manner as
568 described in REF (Deter et al., 2020a)
569
570 **Evolution through cyclic antibiotic treatment.** Stationary phase culture was exposed to 100
571 µg/mL streptomycin (~10× MIC), 1 µg/mL ciprofloxacin (~10× MIC), a combination of
572 streptomycin (100 µg/mL) and ciprofloxacin (1 µg/mL) and 300 µg/mL kasugamycin (~10×
573 MIC) antibiotic for 24 h, then antibiotic-containing medium was removed by washing twice with
574 SP16 medium (10 min of centrifugation at 7000 g at 4°C). Finally, the culture was resuspended
575 in 10 mL of fresh SP16 media and grown overnight at 37 °C. After every cycle of antibiotic
576 treatment, the tolerance phenotype was observed. Finally, we isolated single colony from
577 evolved populations from streptomycin (PS04), ciprofloxacin (PC06), combination of
578 streptomycin and ciprofloxacin (PSC09) and kasugamycin (PK07) treatment after four, six, nine
579 and seven cycle, respectively, and then their genomes were sequenced. Two different
580 evolutionary lineages were used for all evolved populations.
581
582 **Determination of growth and doubling times.** Overnight cultures were diluted into OD 0.1
583 (measured in SpectronicTM 200E) and 30 µL of diluted cultures were inoculated into individual
584 wells containing 270 µL of SP16 media in a 96-Well Optical-Bottom Plate with Polymer Base
585 (ThermoFisher) to measure OD at 600 nm using FLUOstar Omega microplate reader. Doubling

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

586 time was determined by the linear regression of the natural logarithm of the OD over time during
587 exponential growth as described in REF (Widdel, 2007).
588
589 **Statistical analysis.** All data is presented in the manuscript as mean ± SEM of at least three
590 independent biological replicates. Statistical significance was assessed using an f-test to
591 determine variance ($p < 0.05$ was considered to have significant variance), followed by a two-
592 tailed t-test with unequal variances (if F statistic > F critical value) or equal variances (if F
593 statistic < F critical value).
594

595 **Acknowledgments**

596 *Mycoplasma mycoides* JCVI-Syn3B was a generous gift from Dr. John I. Glass from J. Craig
597 Venter Institute (JCVI), La Jolla, CA, USA. This work is supported by the National Science
598 Foundation award Numbers 1922542 and 1849206, and by a USDA National Institute of Food
599 and Agriculture Hatch project grant number SD00H653-18/project accession no. 1015687.
600

601 **Author contributions**

602 T.H. wrote the manuscript and performed most experiments. H.S.D. repeated streptomycin
603 persister assay and developed custom code for colony counting, growth rate, and statistical
604 analyses. E.P. performed cyclic antibiotic treatment experiments. N.C.B. planned and directed
605 the project. All authors contributed to discussing and editing the manuscript.
606

607 **Declaration of interests**

608 The authors declare no competing interests.
609

610 **Data and materials availability**

611 All data that supports the findings of this study are available from the corresponding author upon
612 request. Whole genomes data of P026, PK07_L1, PK07_L2, PS04_L1, PS04_L2, PC06_L1,
613 PC06_L2, PSC09_L1 and PSC09_L2 strains has been deposited on the NCBI Genome Bank in
614 BioProject PRJNA635211 with the accession number CP053944, CP069339, CP069340,
615 CP069341, CP069342, CP069343, CP069344, CP069345, CP069346, respectively. The code
616 used for colony counting is available on GitHub at *https://github.com/hdeter/CountColonies.*
617

618 **References**

619 Abshire, K.Z., and Neidhardt, F.C. (1993). Growth rate paradox of Salmonella typhimurium
620 within host macrophages. Journal of bacteriology *175*, 3744-3748.
621 Aedo, S.J., Orman, M.A., and Brynildsen, M.P. (2019). Stationary phase persister formation in
622 Escherichia coli can be suppressed by piperacillin and PBP3 inhibition. BMC microbiology *19*,
623 1-12.
624 Balaban, N.Q., Helaine, S., Lewis, K., Ackermann, M., Aldridge, B., Andersson, D.I.,
625 Brynildsen, M.P., Bumann, D., Camilli, A., Collins, J.J., *et al.* (2019). Definitions and guidelines
626 for research on antibiotic persistence. Nature Reviews Microbiology *17*, 441-448.
627 Balaban, N.Q., and Liu, J. (2019). Evolution Under Antibiotic Treatments: Interplay Between
628 Antibiotic Persistence, Tolerance, and Resistance. In Persister Cells and Infectious Disease, pp.
629 1-17.
630 Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L., and Leibler, S. (2004). Bacterial persistence as
631 a phenotypic switch. Science *305*, 1622-1625.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

632 Bartell, J.A., Cameron, D.R., Mojsoska, B., Haagensen, J.A.J., Pressler, T., Sommer, L.M.,
633 Lewis, K., Molin, S., and Johansen, H.K. (2020). Bacterial persisters in long-term infection:
634 Emergence and fitness in a complex host environment. PLoS pathogens *16*, e1009112.
635 Bhaskar, A., De Piano, C., Gelman, E., McKinney, J.D., and Dhar, N. (2018). Elucidating the
636 role of (p) ppGpp in mycobacterial persistence against antibiotics. IUBMB life *70*, 836-844.
637 Bigger, J. (1944). TREATMENT OF STAPHYLOCOCCAL INFECTIONS WITH
638 PENICILLIN BY INTERMITTENT STERILISATION. The Lancet *244*, 497-500.
639 Boutte, C.C., and Crosson, S. (2013). Bacterial lifestyle shapes stringent response activation.
640 Trends in microbiology *21*, 174-180.
641 Cabral, D.J., Wurster, J.I., and Belenky, P. (2018). Antibiotic persistence as a metabolic
642 adaptation: stress, metabolism, the host, and new directions. Pharmaceuticals *11*, 14.
643 Cameron, D.R., Shan, Y., Zalis, E.A., Isabella, V., and Lewis, K. (2018). A genetic determinant
644 of persister cell formation in bacterial pathogens. Journal of bacteriology *200*, e00303-00318.
645 Centers for Disease Control and Preventions (2019). Biggest threats and data.
646 Chowdhury, N., Kwan, B.W., and Wood, T.K. (2016). Persistence increases in the absence of the
647 alarmone guanosine tetraphosphate by reducing cell growth. Scientific reports *6*, 20519.
648 Christensen, S.K., Maenhaut-Michel, G., Mine, N., Gottesman, S., Gerdes, K., and Van
649 Melderen, L. (2004). Overproduction of the Lon protease triggers inhibition of translation in
650 Escherichia coli: involvement of the yefM-yoeB toxin-antitoxin system. Molecular microbiology
651 *51*, 1705-1717.
652 Cohen, N.R., Lobritz, M.A., and Collins, J.J. (2013). Microbial persistence and the road to drug
653 resistance. Cell host & microbe *13*, 632-642.
654 Conlon, B.P., Rowe, S.E., Gandt, A.B., Nuxoll, A.S., Donegan, N.P., Zalis, E.A., Clair, G.,
655 Adkins, J.N., Cheung, A.L., and Lewis, K. (2016). Persister formation in Staphylococcus aureus
656 is associated with ATP depletion. Nature microbiology *1*, 1-7.
657 Correia, F.F., D'Onofrio, A., Rejtar, T., Li, L., Karger, B.L., Makarova, K., Koonin, E.V., and
658 Lewis, K. (2006). Kinase activity of overexpressed HipA is required for growth arrest and
659 multidrug tolerance in Escherichia coli. Journal of bacteriology *188*, 8360-8367.
660 Cui, P., Niu, H., Shi, W., Zhang, S., Zhang, W., and Zhang, Y. (2018). Identification of genes
661 involved in bacteriostatic antibiotic-induced persister formation. Frontiers in microbiology *9*,
662 413.
663 Datla, U.S., Mather, W.H., Chen, S., Shoultz, I.W., Täuber, U.C., Jones, C.N., and Butzin, N.C.
664 (2017). The spatiotemporal system dynamics of acquired resistance in an engineered
665 microecology. Scientific reports *7*, 1-9.
666 De la Cruz, M.A., Zhao, W., Farenc, C., Gimenez, G., Raoult, D., Cambillau, C., Gorvel, J.P.,
667 and Meresse, S. (2013). A toxin-antitoxin module of Salmonella promotes virulence in mice.
668 PLoS pathogens *9*, e1003827.
669 Deter, H.S., Abualrahi, A.H., Jadhav, P., Schweer, E.K., Ogle, C.T., and Butzin, N.C. (2019a).
670 Proteolytic queues at ClpXP increase antibiotic tolerance. ACS Synthetic Biology.
671 Deter, H.S., Abualrahi, A.H., Jadhav, P., Schweer, E.K., Ogle, C.T., and Butzin, N.C. (2020a).
672 Proteolytic Queues at ClpXP Increase Antibiotic Tolerance. ACS synthetic biology *9*, 95-103.
673 Deter, H.S., Dies, M., Cameron, C.C., Butzin, N.C., and Buceta, J. (2019b). A Cell
674 Segmentation/Tracking Tool Based on Machine Learning. In Computer Optimized Microscopy
675 (Springer), pp. 399-422.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

676 Deter, H.S., Hossain, T., and Butzin, N.C. (2020b). Antibiotic tolerance is associated with a
677 broad and complex transcriptional response in E. coli. bioRxiv,
678 2020.2008.2027.270272.
679 Dörr, T., Vulić, M., and Lewis, K. (2010). Ciprofloxacin causes persister formation by inducing
680 the TisB toxin in Escherichia coli. PLoS Biol *8*, e1000317.
681 El Meouche, I., and Dunlop, M.J. (2018). Heterogeneity in efflux pump expression predisposes
682 antibiotic-resistant cells to mutation. Science *362*, 686-690.
683 Fisher, R.A., Gollan, B., and Helaine, S. (2017). Persistent bacterial infections and persister cells.
684 Nature Reviews Microbiology *15*, 453.
685 Foster, T.J. (2017). Antibiotic resistance in Staphylococcus aureus. Current status and future
686 prospects. FEMS microbiology reviews *41*, 430-449.
687 Fridman, O., Goldberg, A., Ronin, I., Shoresh, N., and Balaban, N.Q. (2014). Optimization of lag
688 time underlies antibiotic tolerance in evolved bacterial populations. Nature *513*, 418-421.
689 Gaca, A.O., Colomer-Winter, C., and Lemos, J.A. (2015). Many means to a common end: the
690 intricacies of (p) ppGpp metabolism and its control of bacterial homeostasis. Journal of
691 bacteriology *197*, 1146-1156.
692 Garcia Rodriguez, G., Talavera Perez, A., Konijnenberg, A., Sobott, F., Michiels, J., and Loris,
693 R. (2020). The Escherichia coli RnlA–RnlB toxin–antitoxin complex: production,
694 characterization and crystallization. Acta Crystallographica Section F: Structural Biology
695 Communications *76*.
696 Gibson, D.G., Glass, J.I., Lartigue, C., Noskov, V.N., Chuang, R.-Y., Algire, M.A., Benders,
697 G.A., Montague, M.G., Ma, L., and Moodie, M.M. (2010). Creation of a bacterial cell controlled
698 by a chemically synthesized genome. Science *329*, 52-56.
699 Girgis, H.S., Harris, K., and Tavazoie, S. (2012). Large mutational target size for rapid
700 emergence of bacterial persistence. Proceedings of the National Academy of Sciences *109*,
701 12740-12745.
702 Glass, J.I., Merryman, C., Wise, K.S., Hutchison, C.A., 3rd, and Smith, H.O. (2017). Minimal
703 Cells-Real and Imagined. Cold Spring Harb Perspect Biol *9*.
704 Goormaghtigh, F., Fraikin, N., Putrins, M., Hallaert, T., Hauryliuk, V., Garcia-Pino, A., Sjodin,
705 A., Kasvandik, S., Udekwu, K., Tenson, T.*, et al.* (2018). Reassessing the Role of Type II Toxin-
706 Antitoxin Systems in Formation of Escherichia coli Type II Persister Cells. mBio *9*.
707 Hansen, S., Lewis, K., and Vulić, M. (2008). Role of global regulators and nucleotide
708 metabolism in antibiotic tolerance in Escherichia coli. Antimicrobial agents and chemotherapy
709 *52*, 2718-2726.
710 Hansen, S., Vulić, M., Min, J., Yen, T.-J., Schumacher, M.A., Brennan, R.G., and Lewis, K.
711 (2012). Regulation of the Escherichia coli HipBA toxin-antitoxin system by proteolysis. PloS
712 one *7*, e39185.
713 Harms, A., Brodersen, D.E., Mitarai, N., and Gerdes, K. (2018). Toxins, Targets, and Triggers:
714 An Overview of Toxin-Antitoxin Biology. Mol Cell *70*, 768-784.
715 Harms, A., Maisonneuve, E., and Gerdes, K. (2016). Mechanisms of bacterial persistence during
716 stress and antibiotic exposure. Science *354*.
717 Horesh, G., Harms, A., Fino, C., Parts, L., Gerdes, K., Heinz, E., and Thomson, N.R. (2018).
718 SLING: a tool to search for linked genes in bacterial datasets. Nucleic Acids Res.
719 Hu, Y., Kwan, B.W., Osbourne, D.O., Benedik, M.J., and Wood, T.K. (2015). Toxin YafQ
720 increases persister cell formation by reducing indole signalling. Environmental microbiology *17*,
721 1275-1285.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

722 Hutchison, C.A., 3rd, Chuang, R.Y., Noskov, V.N., Assad-Garcia, N., Deerinck, T.J., Ellisman,
723 M.H., Gill, J., Kannan, K., Karas, B.J., Ma, L., *et al.* (2016). Design and synthesis of a minimal
724 bacterial genome. Science *351*, aad6253.
725 Kaldalu, N., Hauryliuk, V., and Tenson, T. (2016). Persisters—as elusive as ever. Applied
726 microbiology and biotechnology *100*, 6545-6553.
727 Kang, S.M., Kim, D.H., Jin, C., and Lee, B.J. (2018). A Systematic Overview of Type II and III
728 Toxin-Antitoxin Systems with a Focus on Druggability. Toxins (Basel) *10*.
729 Karp, P.D., Weaver, D., Paley, S., Fulcher, C., Kubo, A., Kothari, A., Krummenacker, M.,
730 Subhraveti, P., Weerasinghe, D., Gama-Castro, S., *et al.* (2014). The EcoCyc Database. EcoSal
731 Plus *6*, ESP-0009-2013: 0001-0013.
732 Keren, I., Shah, D., Spoering, A., Kaldalu, N., and Lewis, K. (2004). Specialized persister cells
733 and the mechanism of multidrug tolerance in Escherichia coli. Journal of bacteriology *186*, 8172-
734 8180.
735 Kim, J.-S., and Wood, T.K. (2016). Persistent persister misperceptions. Frontiers in
736 microbiology *7*, 2134.
737 Kim, Y., and Wood, T.K. (2010). Toxins Hha and CspD and small RNA regulator Hfq are
738 involved in persister cell formation through MqsR in Escherichia coli. Biochemical and
739 biophysical research communications *391*, 209-213.
740 Kiss, P. (2000). Results concerning products and sums of the terms of linear recurrences. Acta
741 Acad. Agriensis Sectio Math *27*, 1-7.
742 Korch, S.B., Henderson, T.A., and Hill, T.M. (2003). Characterization of the hipA7 allele of
743 Escherichia coli and evidence that high persistence is governed by (p)ppGpp synthesis. Mol
744 Microbiol *50*, 1199-1213.
745 Korch, S.B., and Hill, T.M. (2006). Ectopic overexpression of wild-type and mutant hipA genes
746 in Escherichia coli: effects on macromolecular synthesis and persister formation. Journal of
747 bacteriology *188*, 3826-3836.
748 Kwan, B.W., Valenta, J.A., Benedik, M.J., and Wood, T.K. (2013). Arrested protein synthesis
749 increases persister-like cell formation. Antimicrob Agents Chemother *57*, 1468-1473.
750 Lee, A.J., Wang, S., Meredith, H.R., Zhuang, B., Dai, Z., and You, L. (2018). Robust, linear
751 correlations between growth rates and β-lactam–mediated lysis rates. Proceedings of the National
752 Academy of Sciences *115*, 4069-4074.
753 Lee, J.J., Lee, S.-K., Song, N., Nathan, T.O., Swarts, B.M., Eum, S.-Y., Ehrt, S., Cho, S.-N., and
754 Eoh, H. (2019). Transient drug-tolerance and permanent drug-resistance rely on the trehalose-
755 catalytic shift in Mycobacterium tuberculosis. Nature communications *10*, 1-12.
756 Levin-Reisman, I., Ronin, I., Gefen, O., Braniss, I., Shoresh, N., and Balaban, N.Q. (2017).
757 Antibiotic tolerance facilitates the evolution of resistance. Science *355*, 826-830.
758 Lewin, C.S., and Smith, J.T. (1988). Bactericidal mechanisms of ofloxacin. J Antimicrob
759 Chemother *22 Suppl C*, 1-8.
760 Lewis, K. (2010). Persister cells. Annual review of microbiology *64*, 357-372.
761 Lewis, K. (2012). Persister cells: molecular mechanisms related to antibiotic tolerance. Handb
762 Exp Pharmacol, 121-133.
763 Li, H., and Durbin, R. (2009). Fast and accurate short read alignment with Burrows–Wheeler
764 transform. bioinformatics *25*, 1754-1760.
765 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G.,
766 and Durbin, R. (2009). The sequence alignment/map format and SAMtools. Bioinformatics *25*,
767 2078-2079.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

768 Li, Y., and Zhang, Y. (2007). PhoU is a persistence switch involved in persister formation and
769 tolerance to multiple antibiotics and stresses in Escherichia coli. Antimicrobial agents and
770 chemotherapy *51*, 2092-2099.
771 Liu, J., Gefen, O., Ronin, I., Bar-Meir, M., and Balaban, N.Q. (2020). Effect of tolerance on the
772 evolution of antibiotic resistance under drug combinations. Science *367*, 200-204.
773 Liu, S., Wu, N., Zhang, S., Yuan, Y., Zhang, W., and Zhang, Y. (2017). Variable persister gene
774 interactions with (p) ppGpp for persister formation in Escherichia coli. Frontiers in microbiology
775 *8*, 1795.
776 Lobato-Marquez, D., Moreno-Cordoba, I., Figueroa, V., Diaz-Orejas, R., and Garcia-del Portillo,
777 F. (2015). Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside
778 eukaryotic cells. Sci Rep *5*, 9374.
779 Lobritz, M.A., Belenky, P., Porter, C.B., Gutierrez, A., Yang, J.H., Schwarz, E.G., Dwyer, D.J.,
780 Khalil, A.S., and Collins, J.J. (2015). Antibiotic efficacy is linked to bacterial cellular respiration.
781 Proceedings of the National Academy of Sciences *112*, 8173-8180.
782 Luzzatto, L., Apirion, D., and Schlessinger, D. (1968). Mechanism of action of streptomycin in
783 E. coli: interruption of the ribosome cycle at the initiation of protein synthesis. Proceedings of
784 the National Academy of Sciences of the United States of America *60*, 873.
785 Mariscal, A.M., Kakizawa, S., Hsu, J.Y., Tanaka, K., Gonzalez-Gonzalez, L., Broto, A., Querol,
786 E., Lluch-Senar, M., Pinero-Lambea, C., Sun, L.*, et al.* (2018). Tuning Gene Activity by
787 Inducible and Targeted Regulation of Gene Expression in Minimal Bacterial Cells. ACS
788 synthetic biology *7*, 1538-1552.
789 Meylan, S., Andrews, I.W., and Collins, J.J. (2018). Targeting antibiotic tolerance, pathogen by
790 pathogen. Cell *172*, 1228-1238.
791 Mohiuddin, S.G., Kavousi, P., and Orman, M.A. (2020). Flow-cytometry analysis reveals
792 persister resuscitation characteristics. BMC microbiology *20*, 1-13.
793 Moreno-del Álamo, M., Marchisone, C., and Alonso, J.C. (2020). Antitoxin ε Reverses Toxin ζ-
794 Facilitated Ampicillin Dormants. Toxins *12*, 801.
795 Moyed, H.S., and Bertrand, K.P. (1983). hipA, a newly recognized gene of Escherichia coli K-12
796 that affects frequency of persistence after inhibition of murein synthesis. Journal of bacteriology
797 *155*, 768-775.
798 Mulcahy, L.R., Burns, J.L., Lory, S., and Lewis, K. (2010). Emergence of Pseudomonas
799 aeruginosa strains producing high levels of persister cells in patients with cystic fibrosis. Journal
800 of bacteriology *192*, 6191-6199.
801 Orman, M.A., and Brynildsen, M.P. (2013). Dormancy is not necessary or sufficient for bacterial
802 persistence. Antimicrobial agents and chemotherapy *57*, 3230-3239.
803 Pinel-Marie, M.-L., Brielle, R., Riffaud, C., Germain-Amiot, N., Polacek, N., and Felden, B.
804 (2021). RNA antitoxin SprF1 binds ribosomes to attenuate translation and promote persister cell
805 formation in Staphylococcus aureus. Nature Microbiology, 1-12.
806 Pontes, M.H., and Groisman, E.A. (2019). Slow growth determines nonheritable antibiotic
807 resistance in Salmonella enterica. Sci Signal *12*.
808 Pu, Y., Li, Y., Jin, X., Tian, T., Ma, Q., Zhao, Z., Lin, S.Y., Chen, Z., Li, B., Yao, G.*, et al.*
809 (2019). ATP-Dependent Dynamic Protein Aggregation Regulates Bacterial Dormancy Depth
810 Critical for Antibiotic Tolerance. Mol. Cell *73*, 143-156 e144.
811 Pu, Y., Zhao, Z., Li, Y., Zou, J., Ma, Q., Zhao, Y., Ke, Y., Zhu, Y., Chen, H., and Baker, M.A.
812 (2016). Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. Molecular
813 cell *62*, 284-294.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

814 Riffaud, C., Pinel-Marie, M.-L., and Felden, B. (2020). Cross-Regulations between Bacterial
815 Toxin–Antitoxin Systems: Evidence of an Interconnected Regulatory Network? Trends in
816 Microbiology.
817 Riffaud, C., Pinel-Marie, M.-L., Pascreau, G., and Felden, B. (2019). Functionality and cross-
818 regulation of the four SprG/SprF type I toxin–antitoxin systems in Staphylococcus aureus.
819 Nucleic acids research *47*, 1740-1758.
820 Ronneau, S., and Helaine, S. (2019). Clarifying the link between toxin-antitoxin modules and
821 bacterial persistence. Journal of molecular biology.
822 Sahukhal, G.S., Pandey, S., and Elasri, M.O. (2017). msaABCR operon is involved in persister
823 cell formation in Staphylococcus aureus. BMC microbiology *17*, 218.
824 Sanders, C.C. (1988). Ciprofloxacin: in vitro activity, mechanism of action, and resistance.
825 Reviews of infectious diseases *10*, 516-527.
826 Sassi, M., Augagneur, Y., Mauro, T., Ivain, L., Chabelskaya, S., Hallier, M., Sallou, O., and
827 Felden, B. (2015). SRD: a Staphylococcus regulatory RNA database. Rna *21*, 1005-1017.
828 Schumacher, M.A., Balani, P., Min, J., Chinnam, N.B., Hansen, S., Vulić, M., Lewis, K., and
829 Brennan, R.G. (2015). HipBA–promoter structures reveal the basis of heritable multidrug
830 tolerance. Nature *524*, 59-64.
831 Shenkutie, A.M., Yao, M.Z., Siu, G.K.-h., Wong, B.K.C., and Leung, P.H.-m. (2020). Biofilm-
832 Induced Antibiotic Resistance in Clinical Acinetobacter baumannii Isolates. Antibiotics *9*, 817.
833 Skarstad, K., and Boye, E. (1994). The initiator protein DnaA: evolution, properties and
834 function. Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression *1217*, 111-130.
835 Soltani, M., Vargas-Garcia, C.A., Antunes, D., and Singh, A. (2016). Intercellular Variability in
836 Protein Levels from Stochastic Expression and Noisy Cell Cycle Processes. PLoS Comput Biol
837 *12*, e1004972.
838 Song, S., Gong, T., Yamasaki, R., Kim, J.S., and Wood, T.K. (2019). Identification of a potent
839 indigoid persister antimicrobial by screening dormant cells. Biotechnology and bioengineering
840 *116*, 2263-2274.
841 Song, S., and Wood, T.K. (2020). ppGpp ribosome dimerization model for bacterial persister
842 formation and resuscitation. Biochemical and Biophysical Research Communications *523*, 281-
843 286.
844 Song, S., and Wood, T.K. (2021). Are we really studying persister cells? Environmental
845 Microbiology Reports *13*, 3-7.
846 Sulaiman, J.E., and Lam, H. (2020). Proteomic Investigation of Tolerant Escherichia coli
847 Populations from Cyclic Antibiotic Treatment. J Proteome Res.
848 Sun, C., Guo, Y., Tang, K., Wen, Z., Li, B., Zeng, Z., and Wang, X. (2017). MqsR/MqsA
849 toxin/antitoxin system regulates persistence and biofilm formation in Pseudomonas putida
850 KT2440. Frontiers in microbiology *8*, 840.
851 Trastoy, R., Manso, T., Fernandez-Garcia, L., Blasco, L., Ambroa, A., Del Molino, M.P., Bou,
852 G., Garcia-Contreras, R., Wood, T., and Tomas, M. (2018). Mechanisms of bacterial tolerance
853 and persistence in the gastrointestinal and respiratory environments. Clinical Microbiology
854 Reviews *31*.
855 Tripathi, A., Dewan, P.C., Barua, B., and Varadarajan, R. (2012). Additional role for the ccd
856 operon of F-plasmid as a transmissible persistence factor. Proceedings of the National Academy
857 of Sciences *109*, 12497-12502.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Tsilibaris, V., Maenhaut-Michel, G., Mine, N., and Van Melderen, L. (2007). What Is the Benefit to Escherichia coli of Having Multiple Toxin-Antitoxin Systems in Its Genome? Journal of Bacteriology *189*, 6101-6108.

Tully, J.G., Rose, D.L., Whitcomb, R., and Wenzel, R. (1979). Enhanced isolation of Mycoplasma pneumoniae from throat washings with a newly modified culture medium. Journal of Infectious Diseases *139*, 478-482.

Tuomanen, E., Cozens, R., Tosch, W., Zak, O., and Tomasz, A. (1986). The rate of killing of Escherichia coli byβ-lactam antibiotics is strictly proportional to the rate of bacterial growth. Microbiology *132*, 1297-1304.

Uruén, C., Chopo-Escuin, G., Tommassen, J., Mainar-Jaime, R.C., and Arenas, J. (2021). Biofilms as Promoters of Bacterial Antibiotic Resistance and Tolerance. Antibiotics *10*, 3.

Van den Bergh, B., Michiels, J.E., Wenseleers, T., Windels, E.M., Boer, P.V., Kestemont, D., De Meester, L., Verstrepen, K.J., Verstraeten, N., Fauvart, M.*, et al.* (2016). Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. Nat Microbiol *1*, 16020.

van Gemen, B., Koets, H.J., Plooy, C.A., Bodlaender, J., and Van Knippenberg, P.H. (1987). Characterization of the ksgA gene of Escherichia coli determining kasugamycin sensitivity. Biochimie *69*, 841-848.

Vega, N.M., Allison, K.R., Khalil, A.S., and Collins, J.J. (2012). Signaling-mediated bacterial persister formation. Nature chemical biology *8*, 431-433.

Villellas, C., Aristimuño, L., Vitoria, M.-A., Prat, C., Blanco, S., de Viedma, D.G., Domínguez, J., Samper, S., and Aínsa, J.A. (2013). Analysis of mutations in streptomycin-resistant strains reveals a simple and reliable genetic marker for identification of the Mycobacterium tuberculosis Beijing genotype. Journal of Clinical Microbiology *51*, 2124-2130.

Volkov, I.L., Seefeldt, A.C., and Johansson, M. (2019). Tracking of single tRNAs for translation kinetics measurements in chloramphenicol treated bacteria. Methods *162*, 23-30.

Wang, X., Lord, D.M., Hong, S.H., Peti, W., Benedik, M.J., Page, R., and Wood, T.K. (2013). Type II toxin/antitoxin MqsR/MqsA controls type V toxin/antitoxin GhoT/GhoS. Environmental microbiology *15*, 1734-1744.

Wang, X., and Wood, T.K. (2011). Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. Appl Environ Microbiol *77*, 5577-5583.

Wen, W., Liu, B., Xue, L., Zhu, Z., Niu, L., and Sun, B. (2018). Autoregulation and virulence control by the toxin-antitoxin system SavRS in Staphylococcus aureus. Infection and immunity *86*.

Wen, Y., Behiels, E., and Devreese, B. (2014). Toxin–Antitoxin systems: their role in persistence, biofilm formation, and pathogenicity. Pathogens and disease *70*, 240-249.

Widdel, F. (2007). Theory and measurement of bacterial growth. Di dalam Grundpraktikum Mikrobiologie *4*, 1-11.

Wilmaerts, D., Herpels, P., Michiels, J., and Verstraeten, N. (2019a). Genetic Determinants of Persistence in Escherichia coli. In Persister Cells and Infectious Disease (Springer), pp. 133-180.

Wilmaerts, D., Windels, E.M., Verstraeten, N., and Michiels, J. (2019b). General Mechanisms Leading to Persister Formation and Awakening. Trends Genet *35*, 401-411.

Wood, T.K., and Song, S. (2020). Forming and waking dormant cells: The ppGpp ribosome dimerization persister model. Biofilm *2*, 100018.

Wu, N., He, L., Cui, P., Wang, W., Yuan, Y., Liu, S., Xu, T., Zhang, S., Wu, J., and Zhang, W. (2015). Ranking of persister genes in the same Escherichia coli genetic background demonstrates

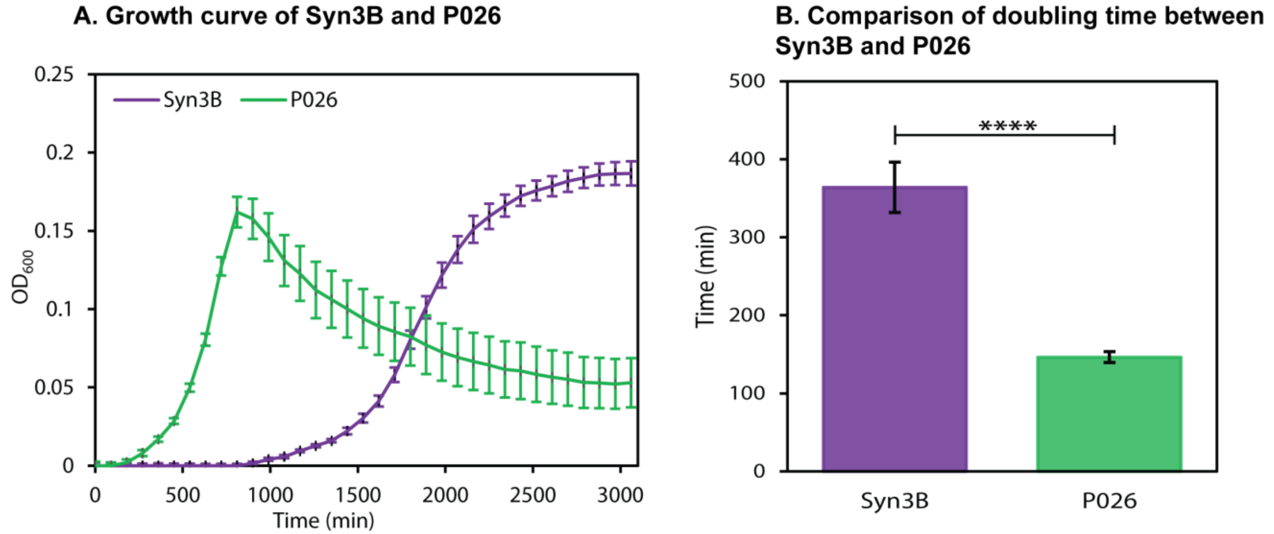
bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

904 varying importance of individual persister genes in tolerance to different antibiotics. Frontiers in
905 microbiology *6*, 1003.
906 Xie, Y., Wei, Y., Shen, Y., Li, X., Zhou, H., Tai, C., Deng, Z., and Ou, H.Y. (2018). TADB 2.0:
907 an updated database of bacterial type II toxin-antitoxin loci. Nucleic Acids Res *46*, D749-D753.
908 Yamasaki, R., Song, S., Benedik, M.J., and Wood, T.K. (2020). Persister cells resuscitate using
909 membrane sensors that activate chemotaxis, lower cAMP levels, and revive ribosomes. Iscience
910 *23*, 100792.
911 Zhang, Y., and Li, Y. (2010). PhoU (PerF), A PERSISTENCE SWITCH INVOLVED IN
912 PERSISTER FORMATION AND TOLERANCE TO MULTIPLE ANTIBIOTICS AND
913 STRESSES AS A DRUG TARGET FOR PERSISTER BACTERIA. (Google Patents).

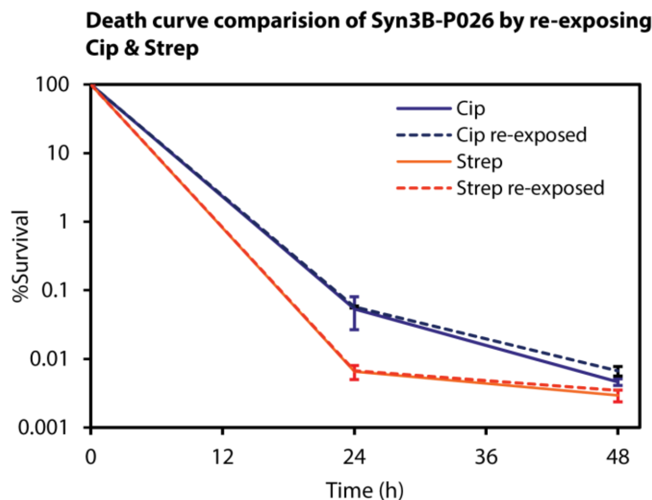
914
915
916

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

917 ## Supplementary materials
918

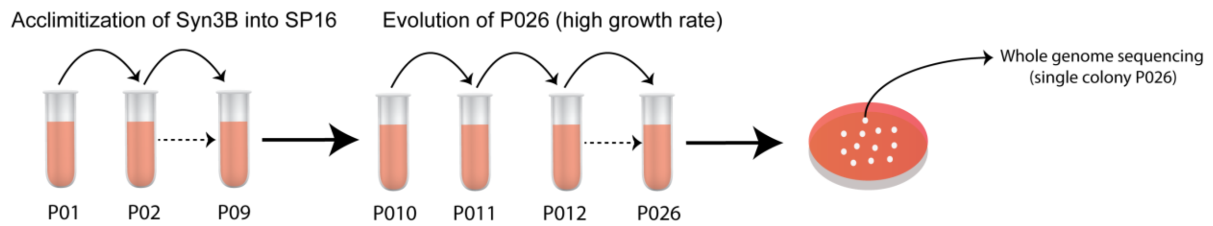


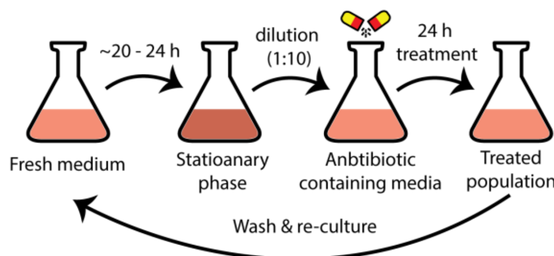
919
920
921 **Fig. S1. Growth of the minimal cell. (A)** Growth curve and **(B)** doubling time of evolved strain
922 Syn3B P026 and parent strain Syn3B. Error bar represents SEM. n = 12 independent biological
923 replicates. *p<0.05, **p<0.01, ***p<0.001, ****p < 0.0001.
924
925



926
927 **Fig. S2. Death curve comparison of Syn3B P026 by re-exposing streptomycin and**
928 **ciprofloxacin.** Overnight cultures of Syn3B were grown to stationary phase (OD~0.3-0.35),
929 diluted to 1:10 and treated with streptomycin (100 µg/mL) or ciprofloxacin (1 µg/mL) for 48 h
930 and sampled after 24 h and 48 h. Antibiotic treated culture then washed twice through
931 centrifugation, grew back to stationary phase and re-exposed to same antibiotic to make the
932 second death curve. Error bars represent SEM (n ≥ 3).

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.





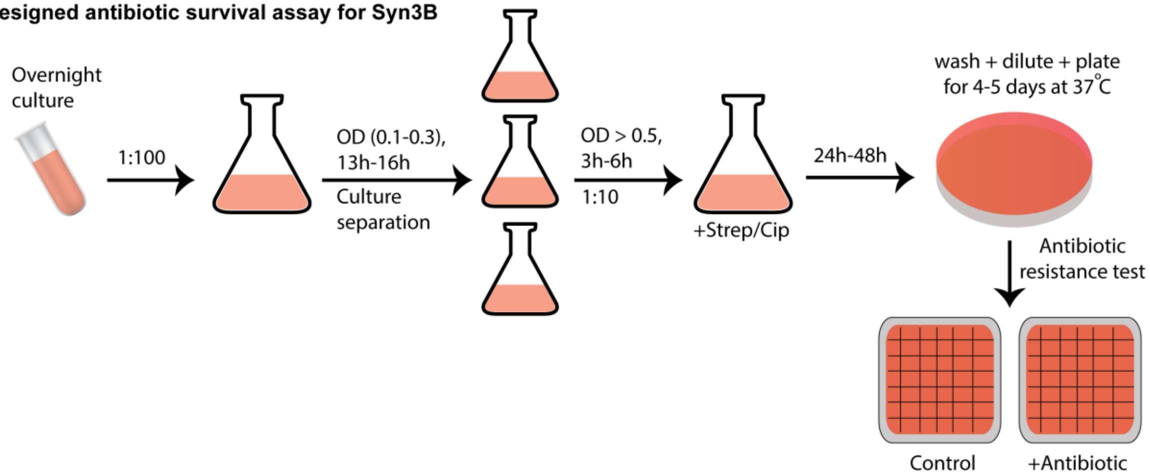
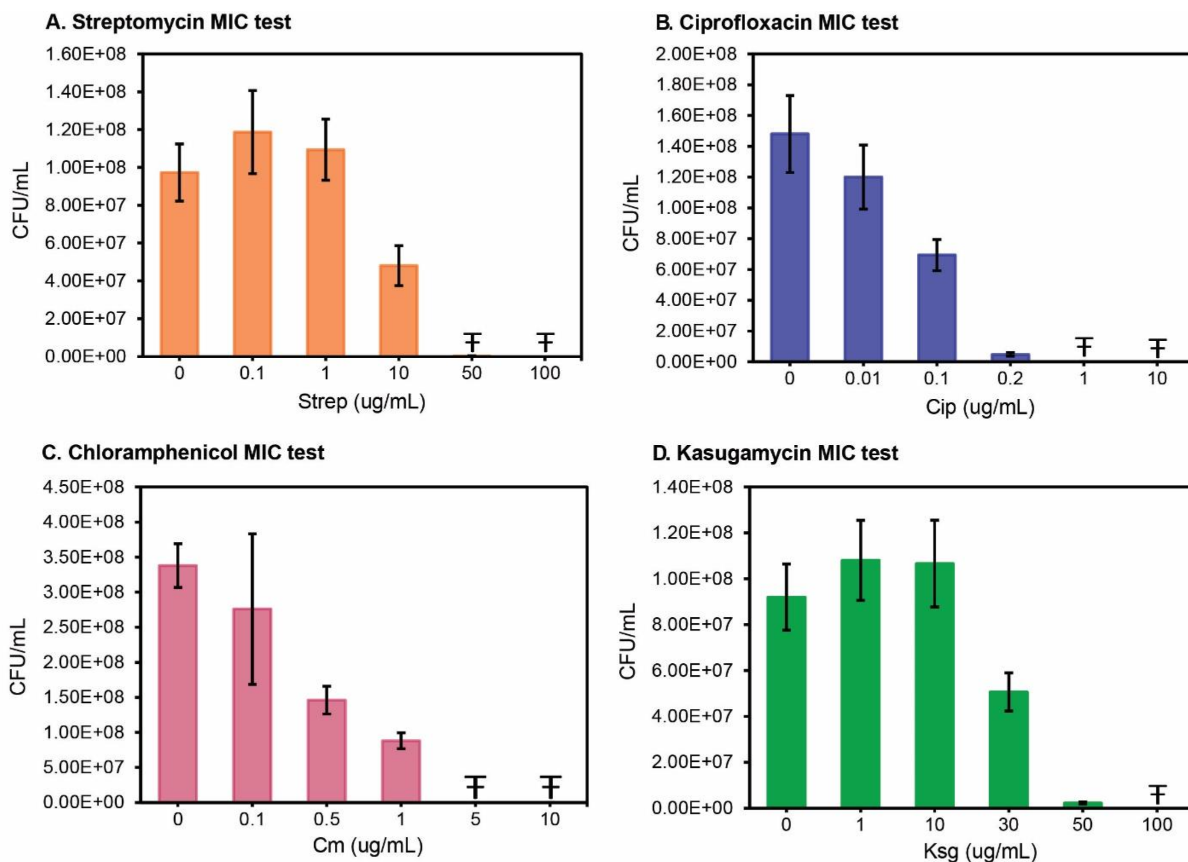


Fig. S3. A. Evolution of Syn3B P026. Cells were evolved in SP16 media by a serial passage from P01 to P09. From P09, exponential cultures were repeatedly diluted 1:100 into fresh SP16 media for higher growth yield up until the 26 passage. Then, a single colony called P026 was selected and send for sequencing. **B.** Evolution of Syn3B resistant mutants through cyclic antibiotic treatment. Stationary phase cultures of P026 were diluted 1:10 in SP16 media containing lethal doses of different types of antibiotic (Streptomycin, Ciprofloxacin, Streptomycin-Ciprofloxacin and Kasugamycin) for 24 h, then washed twice through centrifugation, regrew in the similar condition and re-exposed with same antibiotic. Finally, a single colony was selected for whole genome sequencing. **C.** Antibiotic survival assay was optimized based on traditional agar plate method. Overnight cultures were grown to stationary phase (OD>0.5), diluted to 1:10 in antibiotic-containing media. Percent surviving cells were calculated by the counting colony number before and after antibiotic treatment. Over 200 individual colonies were tested for bacterial resistance, and as expected, no resistant colonies were detected.

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.



950
951 **Fig. S4. Determination of Minimal Inhibitory Concentration (MIC). A.** Streptomycin (Strep)
952 **B.** Ciprofloxacin (Cip). **C.** Chloramphenicol (Cm). **D.** Kasugamycin (Ksg). Exponential phase
953 cultures with different dilutions were plated on SP4 agarose plate with different concentrations
954 of antibiotics. The MIC was determined to be 10 µg/ml for streptomycin, 0.1 µg/mL for
955 ciprofloxacin, 0.5 µg/mL for chloramphenicol, and 30 µg/mL for kasugamycin. Error bars
956 represent the standard deviation and Ŧ represents data is out of detectable range.
957
958
959
960
961
962
963
964
965
966
967
968
969
970
971
972
973

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

974 **Table S1. The** Syn3B genome is lacking homologs to stress response genes that are linked with
975 tolerance and persistence.

Gene name	Major function	Reference
rpoS, fis, hns, hnr, dksA, rob, rcnR	Global regulator	(Cui et al., 2018; Hansen et al., 2008; Wu et al., 2015)
priA, ruvA, recG, recC, recN, uvrD, ybaZ	DNA mismatch repair and recombination	(Cui et al., 2018; Girgis et al., 2012)
recA, lexA, umuD	SOS response	(Cui et al., 2018; Wu et al., 2015)
SpoT	Stringent response	(Korch et al., 2003)
hslU	Protease	(Girgis et al., 2012)
glp, plsB, tkt, sucB, ubiF, ubiE	Energy production	(Girgis et al., 2012)
lpcA, folB, mltC, apaH, surA, ygfA, yigB	Metabolism	(Cui et al., 2018; Hansen et al., 2008)
rfaE, rfaP, rfaQ	Lipopolysaccharide synthesis	(Cui et al., 2018; Girgis et al., 2012)
asmA, tolR	Assembly of outer membrane proteins; maintains outer membrane integrity	(Cui et al., 2018; Girgis et al., 2012)
livJ	Amino acid transporter	(Girgis et al., 2012)
rffM	Enterobacterial common antigen synthesis	(Girgis et al., 2012)
visC	ubiquinone biosynthetic process	(Girgis et al., 2012)
yacC	Exonuclease domain-containing protein	(Girgis et al., 2012)
cspD	DNA replication inhibitor	(Kim and Wood, 2010)
metG	Methionyl-tRNA synthetase	(Girgis et al., 2012)
pspF	Transcriptional activator for the phage shock protein (psp) operon	(Vega et al., 2012)
yiiS, yfcN, yhaC, yjbE, yceA, yagM, ybcK, ydhL, yibA	Unknown	(Cui et al., 2018; Girgis et al., 2012)
flgE, flgJ, fliG, flhB	Flagellar system	(Cui et al., 2018)
rrmJ	Translation	(Cui et al., 2018)
raiA, rmf, hpf, hflX	Ribosome dimerization	(Song and Wood, 2020; Wood and Song, 2020)
hscB, yfhJ	Iron sulfur Cluster	(Cui et al., 2018)
acrA, acrB, yfgL, yfbK	Transporter	(Cui et al., 2018)
oxyR	Antioxidant defense	(Wu et al., 2015)
mqsR-mqsA;dinJ-yafQ;hipA-hipB;yefM-yoeB;rnlB-rnlA;yafN-yafO;mazE-mazF;hicA-hicB;chpS-chpB;higA-higB;prlF-yhaV;relB-relE;vapB-vapC;hok-sok;ldrD-rdlD;tisB-istR;shoB-ohsC;symE-symR;ghoT-ghoS;dinQ-agrB;CbtA-CbtE;parD-parE;ccdA-ccdB;ralR-ralA;zorO-orzO;yeeU-yeeV; pndA-pndB;cptA-cptB;srnB-srnC	TA modules	(Christensen et al., 2004; Dörr et al., 2010; Garcia Rodriguez et al., 2020; Hansen et al., 2012; Hu et al., 2015; Sun et al., 2017; Tripathi et al., 2012; Wang and Wood, 2011; Wen et al., 2014; Wilmaerts et al., 2019a)

976

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

977 **Table S2.** Comparison of % similarity (based on homologous proteins) of Syn3B with most threatening
978 human pathogens.

Human pathogens	Related diseases	Homologous proteins No. in Syn3B*	% similarity with Syn3B
Escherichia coli O157	Severe intestinal infection	287	63
Staphylococcus aureus	Pneumonia, meningitis, osteomyelitis, endocarditis, toxic shock syndrome, bacteremia, sepsis, impetigo, boils, cellulitis, folliculitis and carbuncles etc.	338	74
Burkholderia cepacia	pneumonia in immunocompromised individuals	262	58
Pseudomonas aeruginosa	Urinary tract infections, respiratory system infections and dermatitis	273	60
Clostridium difficile	Diarrhea and inflammation of the colon	329	73
Klebsiella pneumoniae	Pneumonia and infection in the lungs	294	65
Acinetobacter baumannii	Infection in the blood, urinary tract, and lungs (pneumonia)	272	60
Mycobacterium tuberculosis (MTB)	Tuberculosis	275	60
Neisseria gonorrhoeae	Gonorrhea	265	58
Streptococcus pyogenes	Pharyngitis, tonsillitis, scarlet fever, cellulitis, erysipelas and rheumatic fever etc.	328	72

979

980 **Table S3.** Overlapping genes in Syn3B.

Gene name	Gene Length (bp)	Locus tag	Annotation
ieltA	1062	JCVISYN3A_0132	AAA family ATPase
ietS	2,274	JCVISYN3A_0133	hypothetical protein
CDS_33	681	JCVISYN3A_0281	hypothetical protein
proRS	1425	JCVISYN3A_0282	Proline--tRNA ligase
truB	879	JCVSYN2_00715	tRNA pseudouridine(55) synthase TruB
ribF	555	JCVSYN2_00720	FAD synthetase
CDS_50	741	JCVSYN2_00955	hypothetical protein
CDS_51	345	JCVSYN2_00960	hypothetical protein
trmK;\yqfN	678	JCVSYN2_01080	hypothetical protein
CDS_60	777	JCVSYN2_01085	dinuclear metal center protein, YbgI family
pncB	1062	JCVSYN2_01655	Nicotinate phosphoribosyltransferase
CDS_97	609	JCVSYN2_01660	Uncharacterized protein
CDS_124	681	JCVSYN2_02430	hypothetical protein
CDS_125	1539	JCVSYN2_02435	amino acid permease
CDS_99	363	JCVSYN2_01695	lipoprotein
CDS_100	1071	JCVSYN2_01700	hypothetical protein

981
982

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

Table S4. Statistical analysis between different time points of ampicillin death curve for *Escherichia coli* (A), streptomycin death curve for Syn3B P026 (B) and ciprofloxacin death curve for Syn3B P026 (C). Statistical significance was assessed using an f-test to determine variance ($p < 0.05$ was considered to have significant variance), followed by a two-tailed t-test with unequal variances (if F statistic > F critical value) or equal variances (if F statistic < F critical value).

A. *Ampicillin death curve of Escherichia coli*

Time point 1				
24 h	p<0.05	p<0.05	p>0.05	
8 h	p<0.05	p>0.05		p>0.05
6 h	p<0.05		p>0.05	p<0.05
3 h		p<0.05	p<0.05	p<0.05
	3 h	6 h	8 h	24 h

Time point 2

B. *Streptomycin death curve of Syn3B P026*

Time point 1			
72 h	p<0.05	p>0.05	
48 h	p<0.05		p>0.05
24 h		p<0.05	p<0.05
	24 h	48 h	72 h

Time point 2

C. *Ciprofloxacin death curve of Syn3B P026*

Time point 1			
72 h	p<0.05	p>0.05	
48 h	p<0.05		p>0.05
24 h		p<0.05	p<0.05
	24 h	48 h	72 h

Time point 2

Table S5. Experimental variation in population decay curve of Syn3B P026.

Exp. ID	**Streptomycin treatment for 24 h**	**Streptomycin treatment for 48 h**	**Ciprofloxacin treatment for 24 h**	**Ciprofloxacin treatment for 48 h**	**Replicates (n)**
1.	0.03±0.0014	0.0012±0.0003	0.09±0.0096	0.003±0.0005	3
2.	0.083±0.52	0.008±0.067	0.78±0.840	0.017±0.008	3
3.	0.012±0.013	0.006±0.002	3.1±0.93	0.06±0.003	3
4.	0.04±0.009	0.002±0.00012	0.4±0.14	0.007±0.005	3
5.	0.015±0.002	0.008±0.0004	0.5±0.2	0.02±0.014	3
6.	0.004±0.0006	0.002±0002	0.04±0.006	0.004±0006	12

bioRxiv preprint doi: https://doi.org/10.1101/2020.06.02.130641; this version posted March 6, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY-ND 4.0 International license.

1000 **Table S6.** SP16 media composition.

SP16 media components			
Components	**Final**	**Supplier**	**Cat No.**
2XP1			
Mycoplasma Broth Base (PPLO Broth)	7 mg/ml	Thomas Scientific	BD 211458
Tryptone	20 mg/ml	Fisher	AC611841000
Peptone	10.6 mg/ml	Fisher	BP9725-500
Yeast extract solution (autoclaved)	14 mg/ml	Fisher	BP9727-500
TC Yeastolate (Autoclaved)	4 mg/ml	Thomas Scientific	BD 255772
P4			
D(+)-Glucose	0.50%	Fisher	Alfa Aesar A1682836
CMRL 1066 (with L-Glutamine, without Sodium bicarbonate)	0.25X	Thomas Scientific	C992B09 Mfr. No. AT110-1L
NaHCO3 (Sodium Bicarbonate)	1.1 mg/ml	Fisher	S233-3
Penicillin G	625 μg/ml (~1000 U/ml)	Fisher	MP Biomedicals 0210054380 (powder: 500-1700 u/mg)
L-Glutamine	146 μg/ml	Fisher	Alfa Aesar A1420118
FBS			
Fetal Bovine Serum (FBS), Heat inactivated	17%	Fisher	10-438-018 Gibco 10438018
Tetracycline	4 μg/ml	Fisher	BP912-100
Vit B1	5 μg/ml	Acros organic	148990100

1001