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

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Abstract: Antibiotic persisters are a small subpopulation of bacteria that tolerate antibiotics due to a physiologically dormant state. As a result, this phenomenon (persistence) is considered a major contributor to the evolution of antibiotic-resistant and relapsing infections. However, the precise molecular mechanisms of persistence are still unclear. To examine the key mechanisms of persistence, we used the synthetically developed minimal cell *Mycoplasma mycoides* JCVI-Syn3B; the genome contains <500 genes, which are mostly essential. We found that Syn3B evolves expeditiously and rapidly evolves antibiotic resistance to kasugamycin. The minimum cell also tolerates and persists against multiple antibiotics despite lacking many systems related to bacterial persistence (e.g. toxin-antitoxin systems). These results show that this minimal system is a suitable system to unravel the central regulatory mechanisms of persistence.

One Sentence Summary: Essential genes are sufficient for antibiotic tolerance and persistence in the minimal cell.

Main Text:

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 (*1*). 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 (*2, 3*). Tolerance and persistence are non-hereditary phenomena where bacteria enter a dormant state and sustain against antibiotic treatment (*4, 5*). The difference between tolerance and persistence can be seen in a biphasic death curve, where the tolerant population dies off quickly before only persisters remain, dying at a slower rate (*6, 7*). Persisters and tolerant cells can revive and give rise to a new progeny after antibiotic treatment; the new progeny are genetically identical to the original susceptible kin, and this process plays a pivotal role in recurring infections (*8*). Furthermore, evolutionary studies have determined that repeated exposure of antibiotics over many generations rapidly increases persistence leading to antibiotic resistance (*1, 9-12*).

The precise molecular mechanisms underlying persistence are still a riddle, albeit several genes have been implicated (*2, 13, 14*). This riddle is made more complicated because there are two types of persisters, triggered persisters (formed by environmental stimuli) and spontaneous persisters (generated stochastically) (*3*). Toxin-antitoxin (TA) systems have been implicated in both types of persistence and were previously thought to be the key players for persistence (*15-17*). Particularly the HipAB TA system, because a toxin mutant, *hipA7,* increases persistence by impeding cellular growth in the absence of its cognate antitoxin *hipB* (*18*). Subsequent studies

also report that overexpression of other toxins from TA systems increased persistence (*19-21*). In contrast, new research found that the deletion of 10 TA systems in *E. coli* does not have an apparent effect on persistence both at the population and single-cell levels (*22*), though *E. coli* can have more than 45 TA systems (*23-25*). Also, Mauricio *et al.* studied persistence on Δ12TA *Salmonella enterica* and demonstrated TA systems are dispensable (*26*), although this organism has at least 27 bona fide TA systems (*27*).

These findings raise many questions about the implication of TA systems in bacterial persistence (*22, 26, 28*). However, there are major limitations of studying TA systems due to their high abundance in most bacterial genomes; they often have redundant and overlapping functions and can have interdependencies within network clusters (*29, 30*). Additionally, TA systems respond to a variety of stresses (e.g. bacteriophage infection, oxidative stress, etc.), which creates a hurdle to probe their connection with any phenomenon related to stress response, namely antibiotic tolerance and persistence (*29-31*). These challenges could be resolved in a strain that does not contain any TA systems (as we did in this work), but TA systems are naturally abundant and large-scale knockouts are both error-prone and labor-intensive. Furthermore, new types of TA systems may be yet unidentified, and TA systems are also involved in other mechanisms in the cell that respond to stress, such as the stringent and SOS responses (*32*), virulence (*33*), and the regulation of pathogen intracellular lifestyle in varied host cell types (*34*).

Several other persistence mechanisms have been proposed, including the stringent response, SOS response, oxidative stress, and so forth (*8, 13*). Among them, proteases and associated chaperons are notable persister-related genes because overexpression and knockouts of these genes alter persistence levels (*14, 35*). Furthermore, our lab recently demonstrated that interfering with protein degradation by forming a proteolytic queue at ClpXP will increase tolerance levels dramatically and in a tunable fashion (larger queues caused higher tolerance) (*7*). Research over the last several years has resulted in a lot of discussion concerning genes essential for persistence (*8, 13, 26, 29*). Since persistence and tolerance are present in phylogenetically diversified bacterial species (*8*), it is feasible that an underlying genetic mechanism is evolutionarily conserved in evolutionarily related microorganisms, and the most likely candidates are essential genes or the disruption of crucial networks. In this study, we explored antibiotic tolerance, persistence, and resistance in *Mycoplasma mycoides* JCVI-Syn3B (called Syn3B throughout), a synthetic genetic background that contains predominantly essential and a few non-essential genes (*36*). Our work establishes that many systems previously shown to be related to bacterial persistence, such as TA systems, are not vital for antibiotic persistence in the minimal cell, and we demonstrate the effectiveness of using the minimal cell to study antibiotic tolerance and persistence.

Although the minimal genome was designed for ease of genetic manipulation, Syn3B grows slowly and to a low cell density. We adjusted the original SP4 media (*37*) to address these limitations. The new media, SP16, allows for faster cell growth and higher yields in liquid cultures. We noticed that Syn3B growth was slightly better after every subculture. Thus, we did a cyclic subculture of Syn3B in our optimized media by passing cells during logarithmic growth. After 26 passages, we observed better growth and isolated a single colony named Syn3B P026 (Pass 26). This strain has a shorter lag phase and an increased growth rate; the average doubling time of P026 is approximately 2.5 hours compared to the ancestral Syn3B doubling time of ~6

hours under the same conditions (fig. S1A and B). We performed a whole-genome analysis of Syn3B P026 to examine the genetic basis of these changes. Most of the mutations (9 of 11) in P026 are intergenic except one synonymous (*fakA*) and one non-synonymous (*dnaA*) (Table 1). *dnaA* is a positive regulator of DNA replication initiation. Considering that bacterial growth rate is dependent on the frequency of DNA replication and that *dnaA* mutants have been known to result in over-replication (*38*), we hypothesize that the mutation of *dnaA* in P026 is responsible for the higher growth rate. *fakA* is a fatty acid kinase, and there is less evidence to suggest a direct connection to substantial alterations in bacterial growth.

Table 1. Mutation of *Mycoplasma mycoides* JCVI-Syn3B resulted in two strains Syn3B P026 and Syn3B PK15 and Whole Genome Sequence (WGS) analysis identified the mutations (bold and underlined).

Strain	Intergenic mutations	Mutation type	genomic mutation positions[a]	Amino acid substitution	Gene	Annotation
Syn3B P026	9	Nonsynonymous	547	Ala → Thr **G**CA → **A**CA	*dnaA*	Chromosomal replication initiator protein DnaA
		Synonymous	274928	Tyr → Tyr TA**C** → TA**T**	*fakA*	Dihydroxyacetone kinase
Syn3B PK15	8	Nonsynonymous	479174	Pro → Thr **C**CT → **A**CT	*rpoC*	DNA-directed RNA polymerase subunit beta
		Nonsynonymous	3322	Gly → Glu G**G**A → G**A**A	*ksgA*	16S rRNA (adenine(1518)-N(6)/adenine(1519)-N(6))-dimethyltransferase

[a.] Genomic position numberings correspond to *Mycoplasma mycoides* JCVI-Syn3B.

We assessed tolerance and persistence of Syn3B P026 cultures using two bactericidal antibiotics, ciprofloxacin (a fluoroquinolone) and streptomycin (an aminoglycoside). Ciprofloxacin inhibits DNA replication by targeting the activity of DNA gyrase and topoisomerase IV (*39*), whereas streptomycin blocks protein synthesis by irreversibly binding to the 16s rRNA of 30S ribosomal subunit (*40*). P026 was grown to stationary phase and diluted into fresh media containing the antibiotics to observe population decay over 48 hours (see Methods). P026 cultures showed a typical biphasic death curve; the death rate became slower at ~20-24 h treatment with both antibiotics compared to the earlier stage of population decay, which indicates P026 displays both tolerance and persistence (Fig. 1).

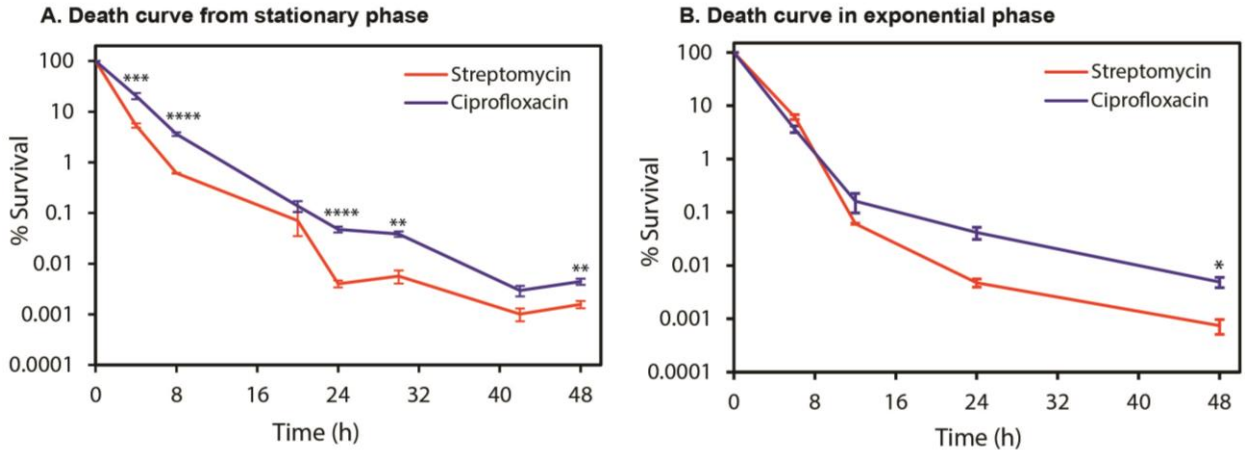

Fig. 1. Population decay during antibiotic treatment. Syn3B P026 cells were treated with streptomycin (100 µg/mL) or ciprofloxacin (1 µg/mL) and sampled after 4 h, 8 h, 20 h, 24 h, 30 h, 42 h, and 48 h. **(A)** Stationary phase cells were diluted 1/10 into fresh media containing antibiotics. Error bars represent SEM ($n \geq 6$). **(B)** Exponential phase cells were treated with antibiotics. 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. P-values are for the difference between antibiotic treatments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$.

We then tested whether we could increase persister levels in P026 using a common persister enrichment method. An established technique to increase bacterial persistence is co-treatment or pre-treatment with a bacteriostatic antibiotic, such as chloramphenicol (*41*, *42*). Chloramphenicol inhibits translation by binding to bacterial ribosomes and inhibiting protein synthesis, thereby inhibiting bacterial growth (*43*). Thus, we co-treated our cultures with the bacteriostatic antibiotic chloramphenicol and either streptomycin or ciprofloxacin and, as expected, the overall percent survival with chloramphenicol co-treatment increased compared to streptomycin or ciprofloxacin alone after 24 h and 48 h (Fig. 2). This result supports that inhibition of translation by co-treating the cell with chloramphenicol increases persister formation in P026 under both types of bactericidal antibiotic treatments.

While we initially quantified triggered persisters (the predominant persister population in stationary phase), we hypothesized that P026 also forms spontaneous persisters. To test for the presence of spontaneous persisters, an exponential phase population was treated with antibiotics, and as expected, we observed a biphasic death (Fig. 1B). The total level of surviving cells were lower for spontaneous compared to triggered persisters for both antibiotic treatments, which is consistent with the literature (*16*). To rule out the possibility of resistance instead of tolerance or persistence, we performed resistance assays through the course of this work (see Methods), and no resistant colonies were identified.

Our results confirm that P026 forms both triggered and spontaneous persisters. However, we also found that in stationary phase, persistence was significantly higher in ciprofloxacin treated populations than in streptomycin treated populations ($p < 0.05$). The higher persistence levels during ciprofloxacin treatment could be due to double-stranded DNA breaks caused by ciprofloxacin activating the SOS response (*44*) (the SOS response is directly related to persistence). We also observed that despite controlling methodology to the greatest extent possible, persister levels varied considerably (far more than observed in our previous work with *E. coli*) (*7*) between experiments for both streptomycin and ciprofloxacin treatments (Table S1). We hypothesize that this variability might be due to the stochastic fluctuations (noise) in gene expression levels, which results in protein level variations significantly even among genetically identical cells in a similar environment (*45*). We expect that gene expression and protein production to be more erratic in Syn3B compared to natural microorganisms because this cell has a designed genome lacking many control mechanisms, and did not have millions of years to achieve some level of internal cellular "equilibrium" like native cells have.

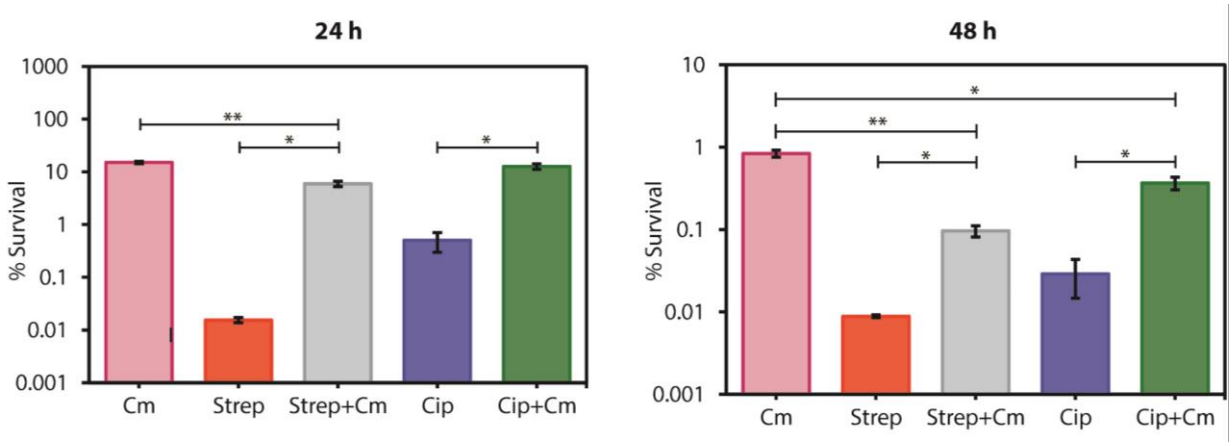

Fig. 2. Co-treatment of bactericidal antibiotic (streptomycin (Strep) or ciprofloxacin (Cip)) with a bacteriostatic antibiotic (chloramphenicol (Cm)) for 24h (left) and 48 h (right) increases triggered persistence in Syn3B P026. Error bars represent SEM ($n \geq 3$). *$p<0.05$. **$p<0.01$.

The mechanisms of persister formation are complex and poorly understood, but recent studies identify several genes involved in this process (*13, 46*). We looked for known and predicted genes related to tolerance and persistence in the Syn3B genome (Table 2). Although TA systems are often implicated for persistence, we did not identify any known TA systems or homologous genes based on the results from TADB 2.0 (a database designed to search for TA systems based on homology) (*47*) in Syn3B. It is not surprising that the genome does not contain TA systems, because the genome was designed by researchers and not subject to the natural environment. Natural TA systems are often thought of as "additive" systems that are hard to lose once acquired, and they often have overlapping toxin and antitoxin genes making mutations less likely to be selected. Curiously, we observed some overlapping genes that are not similar to TA systems (they are also much longer in sequence than a traditional TA system), and whose functions are not yet defined (Table S2).

Table 2. Syn3B contains genes previously shown to be related to *E.coli* persistence/tolerance.

Gene	Protein/RNA	Major function	REF
dnaK, dnaJ, clpB	Chaperon	Global regulator	(*14, 48*)
phoU	Phosphate-specific transport system accessory protein	Global regulator	*(49)*
xseB	Exodeoxyribonuclease	DNA mismatch repair and recombination	(*50*)
uvrA, uvrB	Endonuclease	SOS response	(*14, 50, 51*)
relA	GTP pyrophosphokinase	Stringent response	(*14*)
lon	Protease	Protease	(*14*)
glyA	Serine hydroxymethyltransferase	Metabolism	(*50*)
atpA, atpC, atpF	ATP synthase	ATP synthesis	(*52-54*)
galU	UTP--glucose-1-phosphate uridylyltransferase	Lipopolysaccharide precursor synthesis	(*52*)
trmE; efp; ybeY	tRNA modification GTPase; Elongation factor P; Endoribonuclease	Translation	(*50*)
ssrA; smpB	Transfer messenger RNA (tmRNA); Small Protein B	Trans-translation	(*14*)

A few known persister related genes and pathways are present in Syn3B, including the stringent stress response (*13*). During nutrient limitation, bacteria generally switch their gene expression profile from rapid growth to a survival state by alarmone (p)ppGpp (a hallmark of the stringent response), which is regulated by the RelA protein in *E. coli* (*55*). A potentially fruitful study would be to study the effects on survival by altering (p)ppGpp of Syn3B cultures. Another important gene in stringent response is *phoU*, a global negative regulator, which is deactivated upon inorganic phosphate (Pi) starvation. Mutation or deletion of this gene leads to a metabolically hyperactive status of the cell result in decreased persistence in other microorganisms (*49, 56*). The SOS response is another signaling pathway that upregulates DNA repair functions, which appears to be linked to bacterial persistence. Genes related to the SOS response have been found upregulated in *E. coli* persister (e.g. *uvrA, uvrB*) (*51*) and other mutants (such as *xseB*) have lost their ability to induce persistence when pretreated with rifampicin (*50*). The higher levels of persistence to ciprofloxacin in P026 strongly suggest the SOS response is playing an active role in the survival of the minimal cell. Other findings advocate the connection of persistence with energy metabolism, although the outcomes are often inconsistent (*13*). For example, the deletion of *atpA* (encoding for ATP synthase subunit alpha) decreases the persister fraction (*53*), but deletion of genes encoding other parts of the ATP synthase (*atpC and atpF*) increases the persister fraction (*52, 54*). Several heat shock proteins, mainly proteases (*lon (14, 57)*) and chaperons (*dnaK (14), dnaJ (48), clpB (14)*), are related to persistence; deletion of those genes decrease persistence. Another survival mechanism connected to persistence is trans-translation, which allows bacteria to recycle stalled ribosomes and tag unfinished polypeptides for degradation, which in *E. coli* requires tmRNA (encoded by *ssrA*) and a small protein (smpB). The deletion of these genes causes persister-reduction in *E.coli (14, 58)*. Additional genes (*glyA(50), galU(52), trmE(50), efp(50), ybeY(50)* etc.; see Table 2) are indirectly related to stress response or metabolism and have been reported to affect persistence.

Further exploration is needed to test if there is a relationship between genes in Table 2 to antibiotic survival in the minimal cell.

A key aspect of this work is that there are far fewer genes to explore in Syn3B than any other known free-living microorganism on the planet. It is possible that persister formation and maintenance results from slowed or disrupted cellular networks, rather than the activity of specific genes or regulons. If specific genes or regulons are responsible, then Syn3B should be very useful in identifying them because it has a minimal number of genes. If tolerance/persistence is a result of slowed or disrupted cellular networks, then Syn3B is an ideal model organism to use because of its minimal number of networks. While different genes and networks are likely responsible for persistence depending on antibiotics, strains, or bacterial species, identifying genes in a minimal system should be applicable to other microorganisms including the pathogen *Mycoplasma mycoides*, from which Syn3B was derived.

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). To study the evolution of antibiotic resistance in the minimal genome, we selected kasugamycin (Ksg), a bacteriostatic antibiotic target 16srRNA, and gradually increased the Ksg concentration with every passage (fig. S2.B)., Cultures were continuously passed in SP16 media containing increasing concentrations of Ksg to select for a Ksg resistant mutant. When the culture reached ~50% growth relative to maximum growth without Ksg it was passed into fresh SP16 media with a higher concentration of Ksg. The minimal cell quickly adapted to the Ksg; the population was able to grow at the Minimal Bactericidal Concentration (MBC; 300 µg/mL) of Ksg after only 12 passages. Subsequently, after three additional passages (total of 15 passes) the strain became resistant to a higher level of Ksg (1.1 mg/mL; >3.6X the MBC used to kill the parrent strain) and the growth rate was similar with or without Ksg (fig. S1C). We isolated a single Ksg-resistant colony named Syn3B PK15 (15 Passes in Ksg) and analyzed the whole genome for mutations. Most mutations were in intergenic regions, similar to Syn3B P026, and 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 Ksg causes susceptibility. Therefore, when *ksgA* is inactivated, cells became Ksg-resistant (*59, 60*). We reasoned that the nonsynonymous mutation in *ksgA* in Syn3B PK15 makes the protein inactive and confers resistance to kasugamycin. The other detected non-synonymous mutation is in *rpoC* (RNA polymerase subunit). Interestingly, we observed that the growth rate of PK15 was higher in comparison to ancestor Syn3B (fig. S1A and B), which led us to hypothesize that *rpoC* mutant may also contribute to fast cellular growth like *dnaA* mutant of P026.

Overall, we have demonstrated that 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. In this strain, it is evident that TA systems are not required for bacterial tolerance or persistence. Regardless of how cells enter the dormant state, Syn3B gives us a new approach to study the genes and networks that allow for cells to survive antibiotic treatment and could pave the way of finding new drugs that targets the persister and tolerant subpopulations.

References and Notes:

1. B. Van den Bergh *et al.*, Frequency of antibiotic application drives rapid evolutionary adaptation of Escherichia coli persistence. *Nat Microbiol* **1**, 16020 (2016).
2. N. Q. Balaban, J. Merrin, R. Chait, L. Kowalik, S. Leibler, Bacterial persistence as a phenotypic switch. *Science* **305**, 1622-1625 (2004).
3. E. Maisonneuve, K. Gerdes, Molecular mechanisms underlying bacterial persisters. *Cell* **157**, 539-548 (2014).
4. R. A. Fisher, B. Gollan, S. Helaine, Persistent bacterial infections and persister cells. *Nat. Rev. Microbiol.* **15**, 453-464 (2017).
5. D. J. Cabral, J. I. Wurster, P. Belenky, Antibiotic Persistence as a Metabolic Adaptation: Stress, Metabolism, the Host, and New Directions. *Pharmaceuticals (Basel)* **11**, (2018).
6. N. Q. Balaban *et al.*, Definitions and guidelines for research on antibiotic persistence. *Nature Reviews Microbiology* **17**, 441-448 (2019).
7. H. S. Deter *et al.*, Proteolytic queues at ClpXP increase antibiotic tolerance. *ACS Synthetic Biology*, (2019).
8. D. Wilmaerts, E. M. Windels, N. Verstraeten, J. Michiels, General Mechanisms Leading to Persister Formation and Awakening. *Trends Genet* **35**, 401-411 (2019).
9. O. Fridman, A. Goldberg, I. Ronin, N. Shoresh, N. Q. Balaban, Optimization of lag time underlies antibiotic tolerance in evolved bacterial populations. *Nature* **513**, 418-421 (2014).
10. J. E. Sulaiman, H. Lam, Proteomic Investigation of Tolerant Escherichia coli Populations from Cyclic Antibiotic Treatment. *J Proteome Res*, (2020).
11. N. R. Cohen, M. A. Lobritz, J. J. Collins, Microbial persistence and the road to drug resistance. *Cell host & microbe* **13**, 632-642 (2013).
12. N. Q. Balaban, J. Liu, in *Persister Cells and Infectious Disease*. (2019), chap. Chapter 1, pp. 1-17.
13. D. Wilmaerts, P. Herpels, J. Michiels, N. Verstraeten, in *Persister Cells and Infectious Disease*. (Springer, 2019), pp. 133-180.
14. N. Wu *et al.*, Ranking of persister genes in the same Escherichia coli genetic background demonstrates varying importance of individual persister genes in tolerance to different antibiotics. *Frontiers in microbiology* **6**, 1003 (2015).
15. X. Wang, T. K. Wood, Toxin-antitoxin systems influence biofilm and persister cell formation and the general stress response. *Appl Environ Microbiol* **77**, 5577-5583 (2011).
16. K. Lewis, Persister cells. *Annual review of microbiology* **64**, 357-372 (2010).
17. K. Lewis, Persister cells: molecular mechanisms related to antibiotic tolerance. *Handb Exp Pharmacol*, 121-133 (2012).
18. H. S. Moyed, K. P. Bertrand, hipA, a newly recognized gene of Escherichia coli K-12 that affects frequency of persistence after inhibition of murein synthesis. *Journal of bacteriology* **155**, 768-775 (1983).
19. S. B. Korch, T. M. Hill, Ectopic overexpression of wild-type and mutant hipA genes in Escherichia coli: effects on macromolecular synthesis and persister formation. *Journal of bacteriology* **188**, 3826-3836 (2006).
20. F. F. Correia *et al.*, Kinase activity of overexpressed HipA is required for growth arrest and multidrug tolerance in Escherichia coli. *Journal of bacteriology* **188**, 8360-8367 (2006).
21. Y. Kim, T. K. Wood, Toxins Hha and CspD and small RNA regulator Hfq are involved in persister cell formation through MqsR in Escherichia coli. *Biochemical and biophysical research communications* **391**, 209-213 (2010).

22. F. Goormaghtigh *et al.*, Reassessing the Role of Type II Toxin-Antitoxin Systems in Formation of Escherichia coli Type II Persister Cells. *mBio* **9**, (2018).
23. Y. Xie *et al.*, TADB 2.0: an updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Res.*, (2017).
24. G. Horesh *et al.*, SLING: a tool to search for linked genes in bacterial datasets. *Nucleic Acids Res.*, (2018).
25. P. D. Karp *et al.*, The EcoCyc Database. *EcoSal Plus* **6**, ESP-0009-2013: 0001-0013 (2014).
26. M. H. Pontes, E. A. Groisman, Slow growth determines nonheritable antibiotic resistance in Salmonella enterica. *Sci Signal* **12**, (2019).
27. D. Lobato-Márquez, I. Moreno-Córdoba, V. Figueroa, R. Díaz-Orejas, F. García-del Portillo, Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside eukaryotic cells. *Scientific reports* **5**, 9374 (2015).
28. V. Tsilibaris, G. Maenhaut-Michel, N. Mine, L. Van Melderen, What Is the Benefit to Escherichia coli of Having Multiple Toxin-Antitoxin Systems in Its Genome? *Journal of Bacteriology* **189**, 6101-6108 (2007).
29. S. Ronneau, S. Helaine, Clarifying the link between toxin-antitoxin modules and bacterial persistence. *Journal of molecular biology*, (2019).
30. A. Harms, D. E. Brodersen, N. Mitarai, K. Gerdes, Toxins, Targets, and Triggers: An Overview of Toxin-Antitoxin Biology. *Mol Cell* **70**, 768-784 (2018).
31. S. M. Kang, D. H. Kim, C. Jin, B. J. Lee, A Systematic Overview of Type II and III Toxin-Antitoxin Systems with a Focus on Druggability. *Toxins (Basel)* **10**, (2018).
32. S. Ronneau, S. Helaine, Clarifying the Link between Toxin-Antitoxin Modules and Bacterial Persistence. *Journal of molecular biology*, (2019).
33. M. A. De la Cruz *et al.*, A toxin-antitoxin module of Salmonella promotes virulence in mice. *PLoS pathogens* **9**, e1003827 (2013).
34. D. Lobato-Marquez, I. Moreno-Cordoba, V. Figueroa, R. Diaz-Orejas, F. Garcia-del Portillo, Distinct type I and type II toxin-antitoxin modules control Salmonella lifestyle inside eukaryotic cells. *Sci Rep* **5**, 9374 (2015).
35. W. Wang *et al.*, Transposon mutagenesis identifies novel genes associated with Staphylococcus aureus persister formation. *Frontiers in microbiology* **6**, 1437 (2015).
36. C. A. Hutchison, 3rd *et al.*, Design and synthesis of a minimal bacterial genome. *Science* **351**, aad6253 (2016).
37. J. G. Tully, D. L. Rose, R. Whitcomb, R. Wenzel, Enhanced isolation of Mycoplasma pneumoniae from throat washings with a newly modified culture medium. *Journal of Infectious Diseases* **139**, 478-482 (1979).
38. K. Skarstad, E. Boye, The initiator protein DnaA: evolution, properties and function. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* **1217**, 111-130 (1994).
39. C. C. Sanders, Ciprofloxacin: in vitro activity, mechanism of action, and resistance. *Reviews of infectious diseases* **10**, 516-527 (1988).
40. L. Luzzatto, D. Apirion, D. Schlessinger, Mechanism of action of streptomycin in E. coli: interruption of the ribosome cycle at the initiation of protein synthesis. *Proceedings of the National Academy of Sciences of the United States of America* **60**, 873 (1968).
41. B. W. Kwan, J. A. Valenta, M. J. Benedik, T. K. Wood, Arrested protein synthesis increases persister-like cell formation. *Antimicrob Agents Chemother* **57**, 1468-1473 (2013).
42. C. S. Lewin, J. T. Smith, Bactericidal mechanisms of ofloxacin. *J Antimicrob Chemother* **22 Suppl C**, 1-8 (1988).

43. I. L. Volkov, A. C. Seefeldt, M. Johansson, Tracking of single tRNAs for translation kinetics measurements in chloramphenicol treated bacteria. *Methods* **162**, 23-30 (2019).
44. T. Dorr, K. Lewis, M. Vulic, SOS response induces persistence to fluoroquinolones in Escherichia coli. *PLoS Genet* **5**, e1000760 (2009).
45. M. Soltani, C. A. Vargas-Garcia, D. Antunes, A. Singh, Intercellular Variability in Protein Levels from Stochastic Expression and Noisy Cell Cycle Processes. *PLoS Comput Biol* **12**, e1004972 (2016).
46. D. R. Cameron, Y. Shan, E. A. Zalis, V. Isabella, K. Lewis, A genetic determinant of persister cell formation in bacterial pathogens. *Journal of bacteriology* **200**, e00303-00318 (2018).
47. Y. Xie *et al.*, TADB 2.0: an updated database of bacterial type II toxin-antitoxin loci. *Nucleic Acids Res* **46**, D749-D753 (2018).
48. S. Hansen, K. Lewis, M. Vulić, Role of global regulators and nucleotide metabolism in antibiotic tolerance in Escherichia coli. *Antimicrobial agents and chemotherapy* **52**, 2718-2726 (2008).
49. Y. Li, Y. Zhang, PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in Escherichia coli. *Antimicrobial agents and chemotherapy* **51**, 2092-2099 (2007).
50. P. Cui *et al.*, Identification of genes involved in bacteriostatic antibiotic-induced persister formation. *Frontiers in microbiology* **9**, 413 (2018).
51. I. Keren, D. Shah, A. Spoering, N. Kaldalu, K. Lewis, Specialized persister cells and the mechanism of multidrug tolerance in Escherichia coli. *Journal of bacteriology* **186**, 8172-8180 (2004).
52. H. S. Girgis, K. Harris, S. Tavazoie, Large mutational target size for rapid emergence of bacterial persistence. *Proceedings of the National Academy of Sciences* **109**, 12740-12745 (2012).
53. M. A. Lobritz *et al.*, Antibiotic efficacy is linked to bacterial cellular respiration. *Proceedings of the National Academy of Sciences* **112**, 8173-8180 (2015).
54. P. Kiss, Results concerning products and sums of the terms of linear recurrences. *Acta Acad. Agriensis Sectio Math* **27**, 1-7 (2000).
55. C. C. Boutte, S. Crosson, Bacterial lifestyle shapes stringent response activation. *Trends in microbiology* **21**, 174-180 (2013).
56. Y. Zhang, Y. Li. (Google Patents, 2010).
57. Y. Pu *et al.*, Enhanced efflux activity facilitates drug tolerance in dormant bacterial cells. *Molecular cell* **62**, 284-294 (2016).
58. S. Liu *et al.*, Variable persister gene interactions with (p) ppGpp for persister formation in Escherichia coli. *Frontiers in microbiology* **8**, 1795 (2017).
59. B. van Gemen, H. J. Koets, C. A. Plooy, J. Bodlaender, P. H. Van Knippenberg, Characterization of the ksgA gene of Escherichia coli determining kasugamycin sensitivity. *Biochimie* **69**, 841-848 (1987).
60. A. M. Mariscal *et al.*, Tuning Gene Activity by Inducible and Targeted Regulation of Gene Expression in Minimal Bacterial Cells. *ACS Synth Biol* **7**, 1538-1552 (2018).
61. Li, H. & Durbin, R. Fast and accurate short read alignment with Burrows–Wheeler transform. *Bioinformatics* **25**, 1754-1760 (2009).
62. Li, H. et al. The sequence alignment/map format and SAMtools. *Bioinformatics* **25**, 2078-2079 (2009).

63. I. Levin-Reisman et al., Antibiotic tolerance facilitates the evolution of resistance. *Science* **355**, 826-830 (2017).
64. U. S. Datla et al., The spatiotemporal system dynamics of acquired resistance in an engineered microecology. *Sci Rep* **7**, 16071 (2017).
65. H. S. Deter, M. Dies, C. C. Cameron, N. C. Butzin, J. Buceta, in Methods Mol Biol, R. E., B. M., Eds. (Humana, New York, NY, 2019), vol. 2040, pp. 399-422.

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Supplementary Materials:

Materials and Methods

Figures S1 to S3

Tables S1 to S3

References *(61-65*)

Materials and Methods

Microbial strains and media. *Mycoplasma mycoides* JCVI-Syn3B (*36*) and its derivatives were used in this study. Syn3B was a gift from Dr. John I. Glass from J. Craig Venter Institute, La Jolla, CA, USA. For evolution and antibiotic survival assays, cells were cultured at 37˚C in SP16 media (57.5% 2X P1, 10.0% P4, 17.0% FBS, tetracycline 0.4% and vitamin B1 0.5%) (see **Table S3** for details), which was developed based on SP4 media (*37*). All cultures were plated in SP4 agarose media (0.55% agarose) for colony counts. Note that agar is not used because it inhibits growth.

Evolution by serial passage. Syn3B were grown in 3 mL tubes at 37 ˚C overnight in SP16 media. Overnight exponential cultures were serially passaged after each cycle of growth by transferring 30 µL of culture into 3 mL fresh media to initiate the next cycle of growth and the cycles continued until a satisfactory growth rate was observed (Fig. S2.A). The cultures were plated after 26 passages and a single colony was isolated, P026. For the selection of Ksg-resistant mutants, cells from the 9th passage were grown for 20 to 24 hours at 37˚C in both SP16 and SP16+ Ksg media. Ksg concentration ranged from 15 µg/mL to 1.1 mg/mL. When the culture reached at least 50% growth (OD 0.2-0.25) relative to maximum growth in SP16 alone were passed into a fresh SP16 media + Ksg. A total of 15 serial passages were performed to generate the kasugamycin-resistant strain. (Fig. S2.B)

Genome extraction, whole-genome sequencing, and identification of mutations. For whole-genome sequencing (WGS), a single colony of the evolved strains Syn3B P026 and PK15 were isolated and inoculated into SP16 media for 24 h at 37˚C. Next, genomic DNA was harvested and purified using Genomic DNA Purification Kit (ThermoFisher) in accordance with manufacturer's instructions. For quality checks, DNA purity and concentration were assessed by gel electrophoresis and Qubit Fluorimeter prior to sending for sequencing.
Novogene Ltd. Sequenced the genomes using paired-end Illumina sequencing at 2 × 150 bp read length and 350 bp insert size. A total amount of ~1 µg of DNA per sample was used as input material for the DNA sample preparation. Sequencing libraries were generated from the qualified DNA samples(s) using the NEB Next Ultra DNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's protocol. For data processing, the original sequence data were transformed into raw sequenced reads by CASAVA base calling and stored in FASTQ (fq) format. For subsequent analysis, the raw data were filtered off the reads containing adapter and low-quality reads to obtain clean data. The resequencing analysis was based on reads mapping to reference genome of Syn3B by BWA software (*61*). SAMTOOLS (*62*) was used to detect single nucleotide polymorphism (SNP) and InDels. The mapping rates were found 98.48 and 99.40 for P026 and PK15, respectively.
To search for large insertions or gene duplication in P026 and PK15, we also used Oxford Nanopore Technologies (ONT) MinION long-read sequencer. ONT libraries were prepared using *Ligation kit* (SQK-LSK109) according to the manufacturer's instructions. R 9.5 flow cell (FLO-MIN107, ONT) was used for sequencing based on manufacture protocol. The flow cell was mounted on a MinION Mk 1B device (ONT) for sequencing with the MinKNOW versions 19.12.5_Sequencing_Run_FLO-MIN107_SQK-LSK109 script. Then, reads were mapped against reference genome Syn3B using Geneious Prime software version 2020.1. (*https://www.geneious.com*). No large insertions were found in the sequenced genomes.

Minimum inhibitory concentration (MIC) tests: To determine the MIC of each antibiotic, overnight cultures were serially diluted and plated onto SP4 agarose plates containing different concentrations of antibiotics (Strep, Cip, Cm, and Ksg). Plates were incubated 4-5 days at 37°C before colony counts. MIC values were defined in this study as the lowest antibiotic concentration where no growth was observed (See Fig. S3).

Antibiotic survival assays. The schematic of the antibiotic survival assay from a stationary phase culture is shown in Fig. S2. C. Briefly, an overnight culture was diluted 1:100 into pre-warmed media and grown to exponential phase (OD_{600} 0.1-0.3). Next, the culture was separated into three flasks (for three biological replicates) and grown at 37˚C until it reached stationary phase (OD 0.45-0.55, which takes ~3-6 h). After that, each culture was diluted 1:10 into 100 µg/mL streptomycin (10X MIC) or 1 µg/mL (10X MIC) ciprofloxacin containing pre-warmed media and kept at 37˚C shaking at 250 rpm for 48 h. Samples were taken at different time points until 48 h for the time-kill assays (Fig. 1). To remove the antibiotic before plating, 100 µl of each sample was washed with 1.9 mL ice-cold SP16 media and collected by centrifugation (16,000 rpm for 3 min at 4˚C). Cells were then resuspended and serially diluted into ice-cold SP16 media and plated to count the colony-forming units (CFU). Persisters were quantified by comparing CFUs per milliliter (CFU/ml) before antibiotic treatment to CFU/ml after antibiotic treatment. Plates were incubated at 37ºC for 4-5 days, then scanned using a flatbed scanner (*7, 63-64*). Custom scripts were used to identify and count bacterial colonies (*64-65*) used to calculate CFU/ml and persister frequency. Over 200 colonies were streaked periodically into antibiotic-containing plates to test for resistance.

Determination of growth and doubling times

Overnight cultures were diluted into OD 0.1 (measured in SpectronicTM 200E) and 30 µL of diluted cultures were inoculated into individual wells containing 270 µL of SP16 media in a 96-Well Optical-Bottom Plate with Polymer Base (ThermoFisher) to measure OD at 600 nm using FLUOstar Omega microplate reader. Doubling time was determined by the linear regression of the natural logarithm of the OD over time during exponential growth as described in REF (*6)*.

Statistical analysis. All data is presented in the manuscript as mean ± SEM of at least three independent biological replicates. 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).

Supplementary Figures

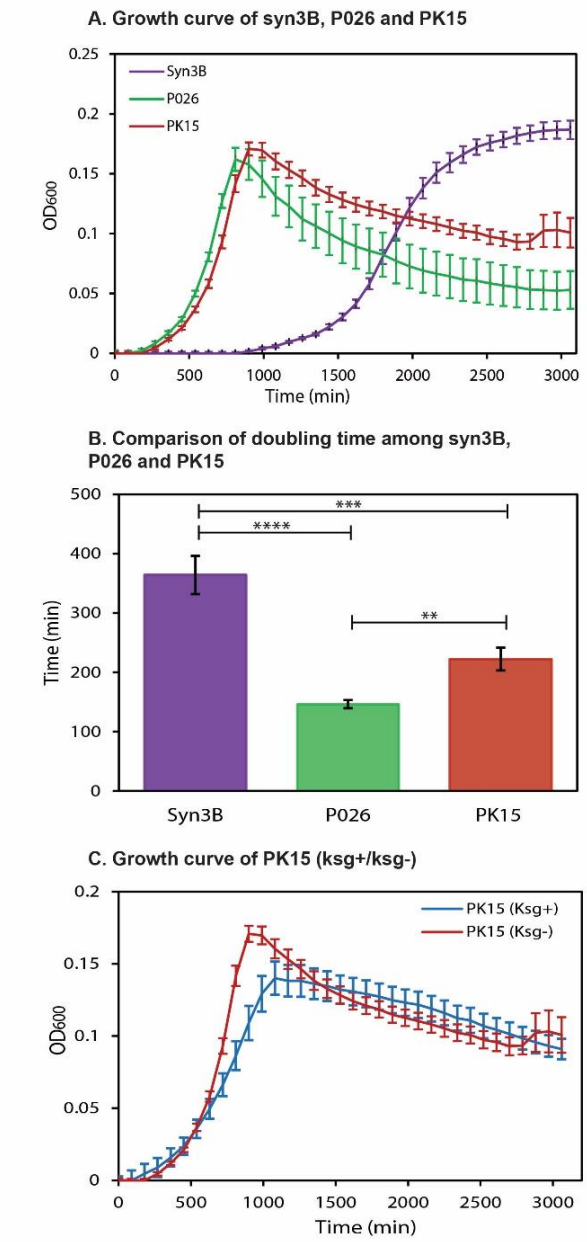

Fig. S1. Growth of the minimal cell. (A) Growth curve and **(B)** doubling time of evolved strain Syn3B P026 and PK15 and parent strain Syn3B. **(C)** Growth curve of Ksg-resistant strain PK15 without (-) and with (+) kasugamycin (Ksg; 500 µg/mL). Section for Ksg-mutants had Ksg (500 µg/mL) in the media. No growth defect was found in PK15 with or without Ksg ($p > 0.05$). Error bar represents SEM. n = 12 independent biological replicates. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p < 0.0001$.

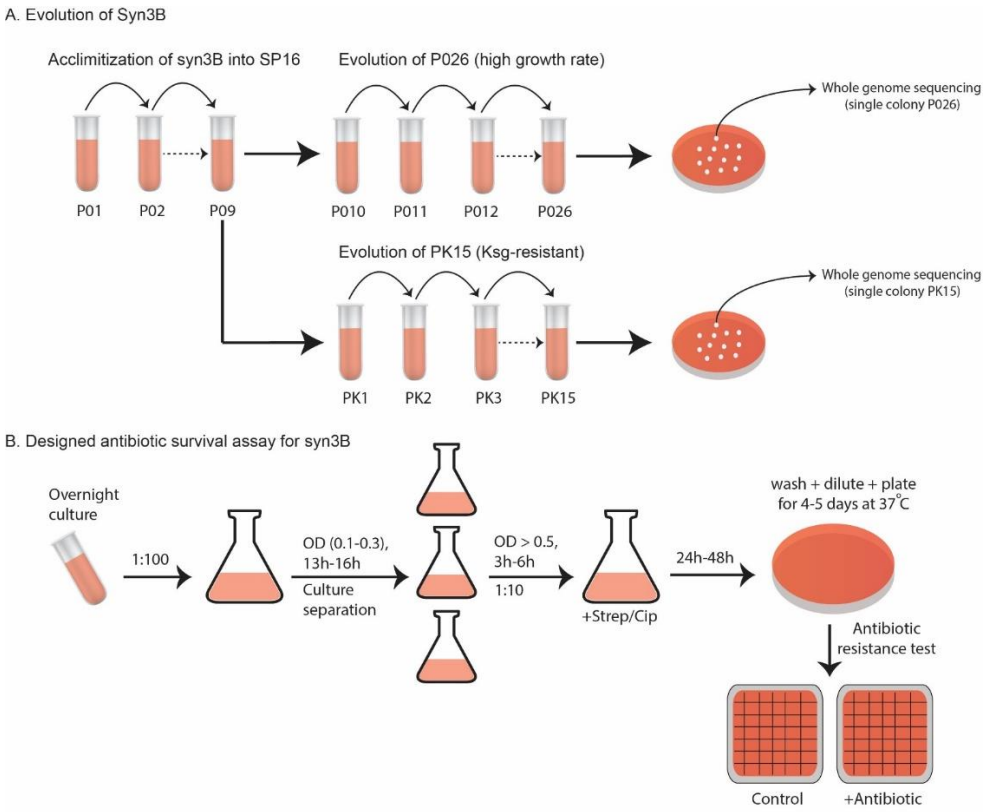

Fig. S2. Methods. A. Evolution of Syn3B: Cells were evolved in SP16 media by a serial passage from P01 to P09. From P09, exponential cultures were repeatedly diluted 1:100 into fresh SP16 media for higher growth yield up until the 26 passage. Then, a single colony of called P026 was selected and send for sequencing. Evolution of PK-15: Exponential cultures of P09 were diluted 1:100 in SP16 and SP16+ksg media. Ksg concentration was gradually increased. A single colony called PK15 (Pass Ksg 15 times) strain was selected and genome sequenced. **B.** 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.

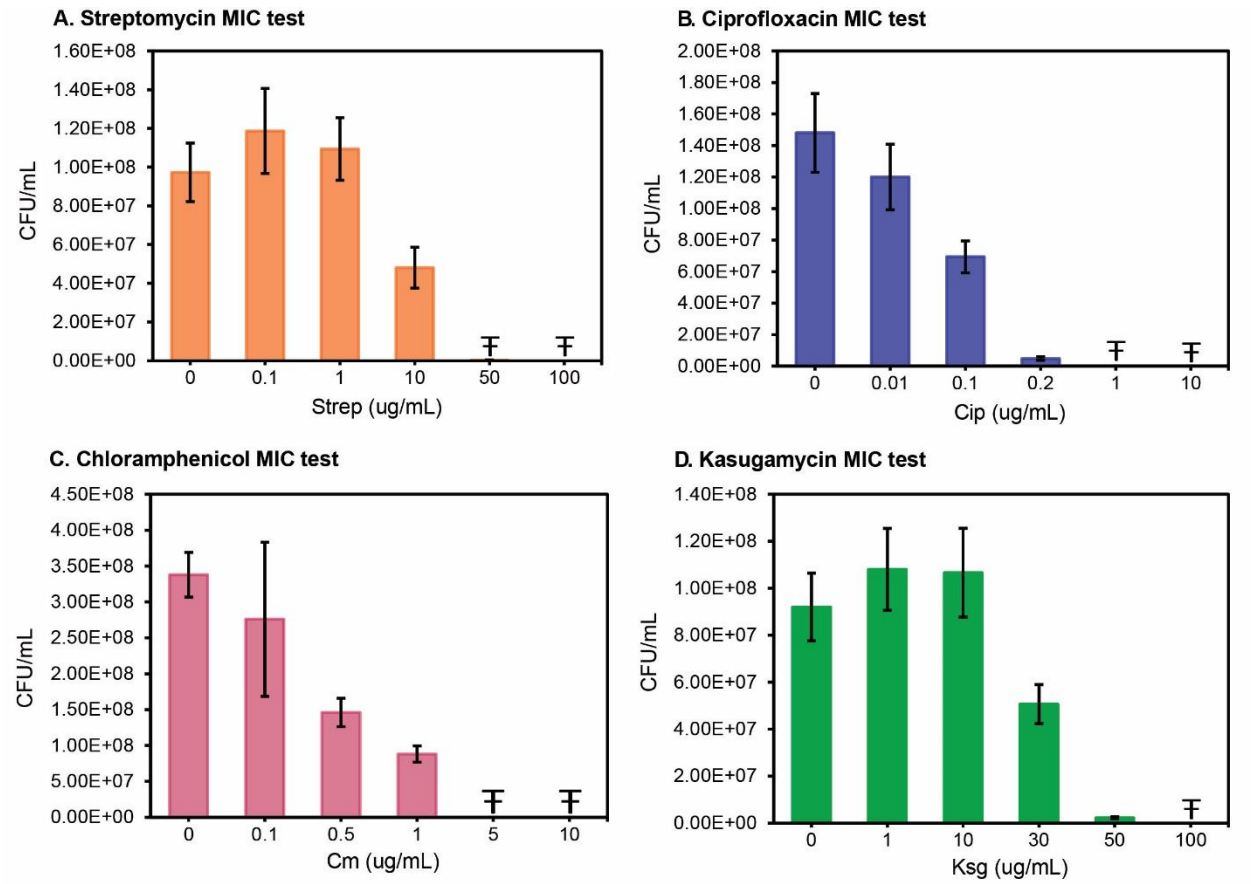

Fig. S3. Determination of Minimal Inhibitory Concentration (MIC). A. Streptomycin (Strep) **B.** ciprofloxacin (Cip). **C.** Chloramphenicol (Cm). **D.** Kasugamycin (Ksg). Exponential phase cultures with different dilutions were plated in different concentrations of antibiotic-containing SP4 agarose plate. The MIC was determined to be 10 µg/ml for streptomycin, 1 µg/mL for ciprofloxacin, 5 µg/mL for chloramphenicol, and 30 µg/mL for kasugamycin. Error bars represent the standard deviation and Ŧ represents data is out of detectable range.

Supplementary Tables

Table S1. 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

Table S2. Overlapping genes in Syn3B. Overlapping genes have the same color.

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

Table S3. 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 (Expensive)	17%	Fisher	10-438-018 Gibco 10438018
Tetracycline	4 µg/ml	Fisher	BP912-100
Vit B1	5 µg/ml	Acros organic	148990100