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Supplemental information

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

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Supplemental materials:

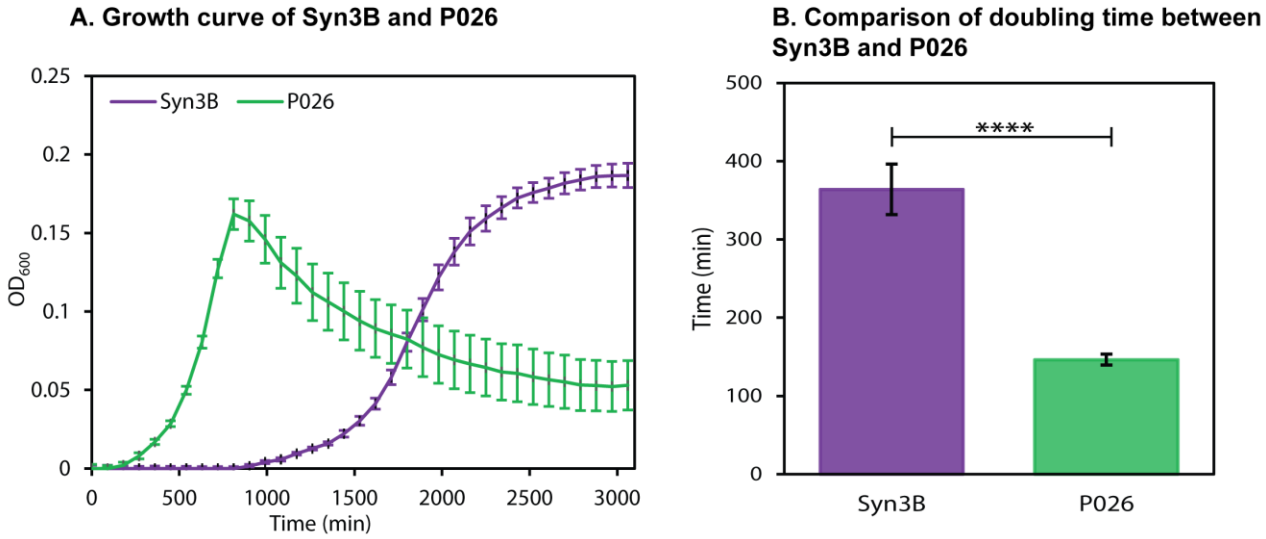

Figure S1. Growth of the minimal cell. Related to Figure 2 and Table 1. (A) Growth curve and **(B)** doubling time of evolved strain Syn3B P026 and parent strain Syn3B. Error bar represents SEM. n = 12 independent biological replicates. *p<0.05, **p<0.01, ***p<0.001, ****p < 0.0001.

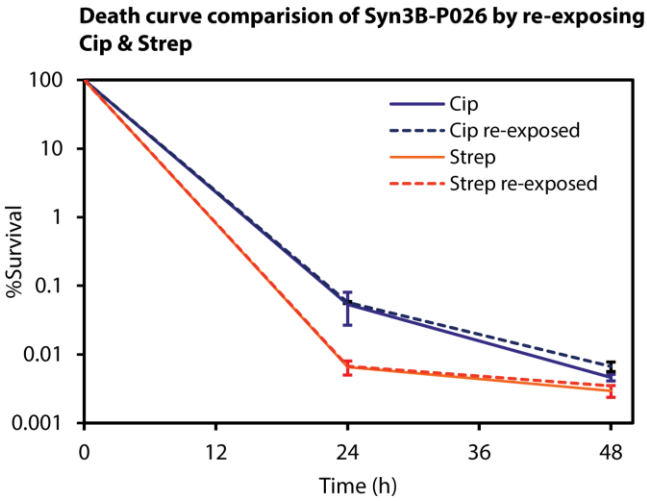

Figure S2. Death curve comparison of Syn3B P026 by re-exposing streptomycin and ciprofloxacin. Related to Figure 2. Overnight cultures of Syn3B were grown to stationary phase (OD~0.3-0.35), diluted to 1:10 and treated with streptomycin (100 µg/mL) or ciprofloxacin (1 µg/mL) for 48 h and sampled after 24 h and 48 h. Antibiotic treated culture then washed twice through centrifugation, grew back to stationary phase and re-exposed to same antibiotic to make the second death curve. Error bars represent SEM (n ≥ 3).

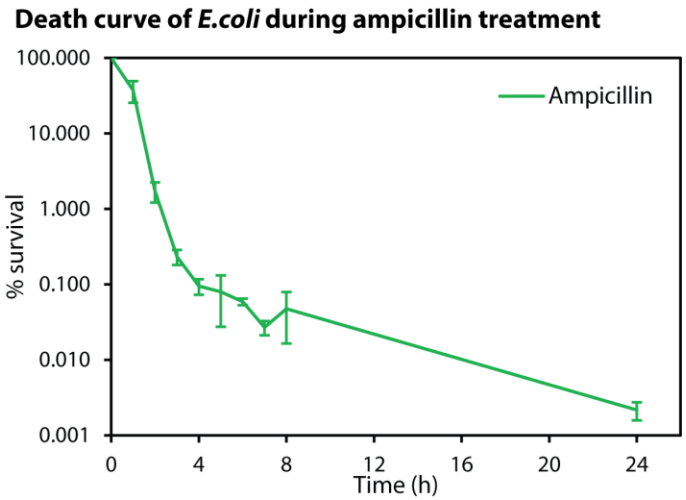

Figure S3. Death curve of *E.coli* during ampicillin treatment. Related to Figure 2. Stationary phase cells were diluted 1/100 into fresh media containing ampicillin (100 μg/mL) and sampled every hour for 8 h ($n \geq 3$) and this result has been published (Deter et al., 2020). In this study, we reanalyzed the data including 24 h treatment data (which was done in similar manner as described in (Deter et al., 2020)) to observe the slow decline in the death curve.

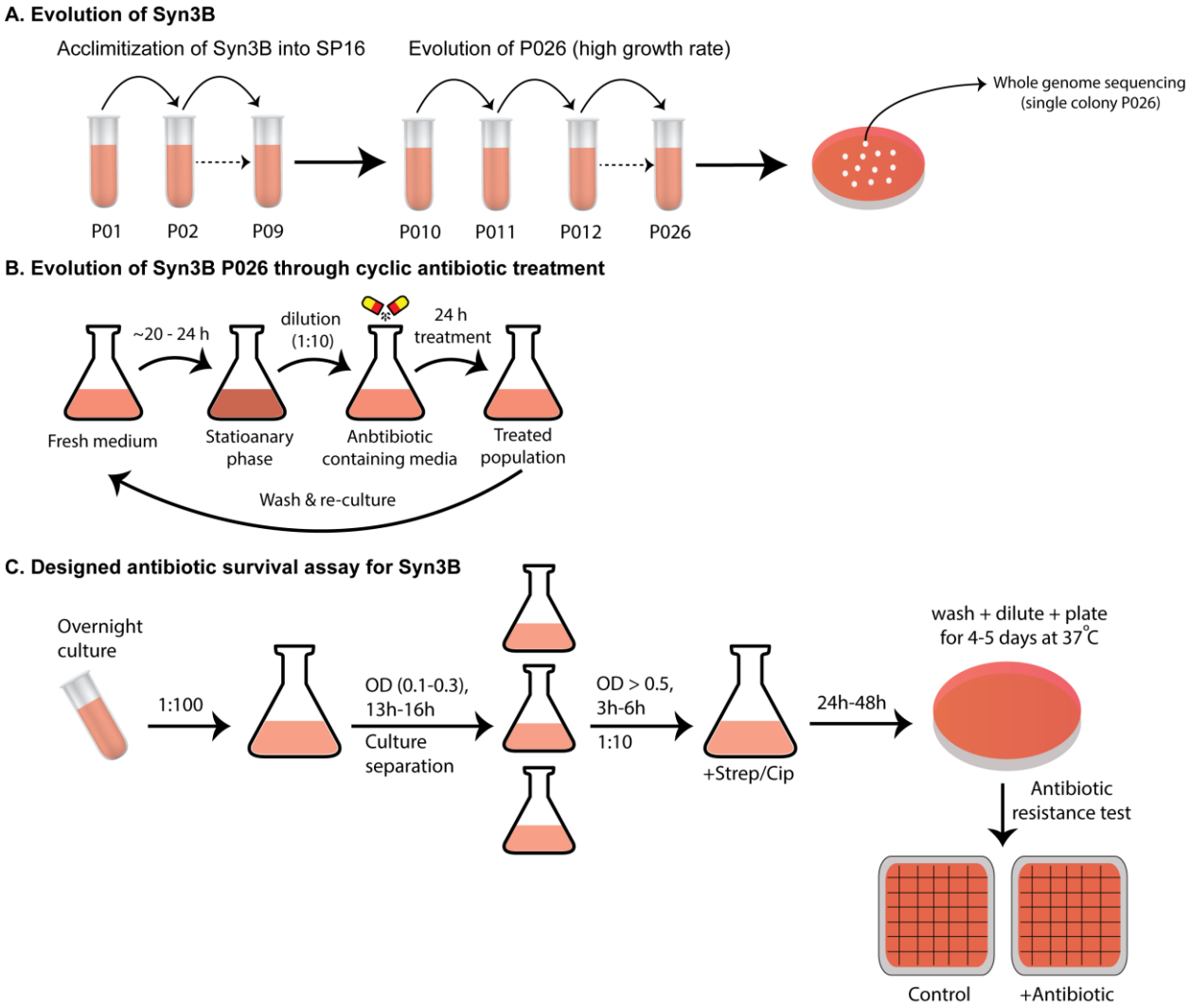

Figure S4. Methods. Related to Figure 2 and Table 1. A. Evolution of Syn3B P026. Cells were evolved in SP16 media by a serial passage from P01 to P09. From P09, exponential cultures were repeatedly diluted 1:100 into fresh SP16 media for higher growth yield up until the 26 passage. Then, a single colony 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 antibiotics (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.

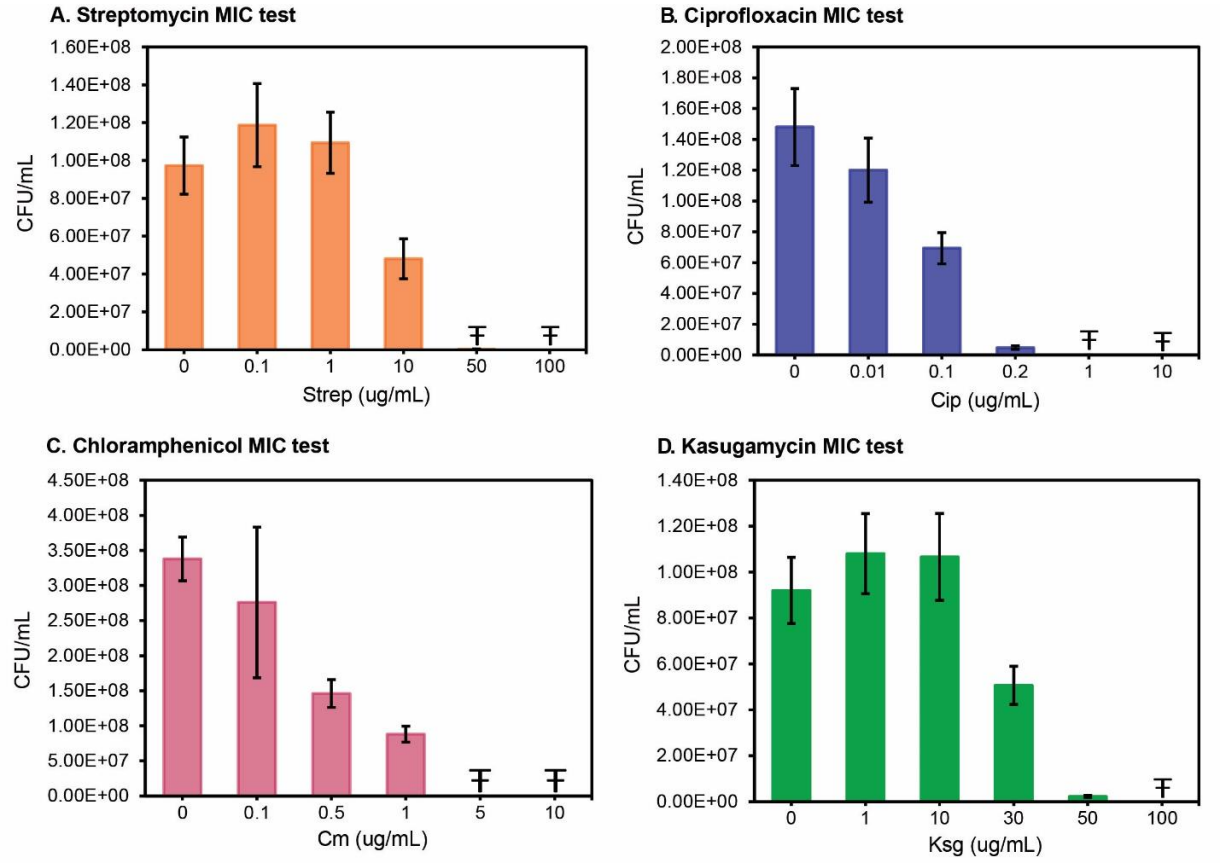

Figure S5. Determination of Minimal Inhibitory Concentration (MIC). Related to Figure 2, 3 and Table 1. A. Streptomycin (Strep) **B.** Ciprofloxacin (Cip). **C.** Chloramphenicol (Cm). **D.** Kasugamycin (Ksg). Exponential phase cultures with different dilutions were plated on SP4 agarose plate with different concentrations of antibiotics. The MIC was determined to be 10 μg/ml for streptomycin, 0.1 μg/mL for ciprofloxacin, 0.5 μg/mL for chloramphenicol, and 30 μg/mL for kasugamycin. Error bars represent the standard deviation and ∓ represents data is out of detectable range.

Table S1. The Syn3B genome is lacking homologs to stress response genes that are linked with tolerance and persistence. Related to Table 2.

Gene name	Major function	Reference
rpoS, fis, hns, hnr, dksA, rob, rcnR	Global regulator	(Wu et al., 2015, Cui et al., 2018, Hansen et al., 2008)
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	(Wu et al., 2015, Cui et al., 2018)
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	(Girgis et al., 2012, Cui et al., 2018)
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	(Girgis et al., 2012, Cui et al., 2018)
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	(Sun et al., 2017, Hu et al., 2015, Dörr et al., 2010, Hansen et al., 2012, Christensen et al., 2004, Tripathi et al., 2012, Garcia Rodriguez et al., 2020, Wen et al., 2014, Wilmaerts et al., 2019, Wang and Wood, 2011)

Table S2. Comparison of % similarity (based on homologous proteins) of Syn3B with most threatening human pathogens. These pathogens can form persisters which leads to antibiotic resistance. Related to Figure 1.

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

Table S3. 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). Related to Figure 2.

A. *Ampicillin death curve of Escherichia coli*

Time point				
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. *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

C. *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

Table S4. Overlapping genes in Syn3B. Related to Table 2.

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 S5. Experimental variation in population decay curve of Syn3B P026. Related to Figure 2.

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 S6. SP16 media composition. Related to Figure 2, 3 and Table 1.

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

Transparent methods:

Microbial strains and media. *Mycoplasma mycoides* JCVI-Syn3B (Hutchison et al., 2016) 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 S6 for details), which was developed based on SP4 media (Tully et al., 1979). 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 cultures 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 (Figure S4. A). The culture was plated after 26 passages and a single colony was isolated, P026.

Genome extraction, whole-genome sequencing, and identification of mutations. For whole-genome sequencing (WGS), a single colony of the evolved strains Syn3B P026 and all the resistant mutants (PK07_L1, PK07_L2, PS04_L1, PS04_L2, PC06_L1, PC06_L2, PSC09_L1, PSC09_L2) 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 the 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 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 (Li and Durbin, 2009). SAMTOOLS (Li et al., 2009) was used to detect single nucleotide polymorphism (SNP) and InDels.

We also used Oxford Nanopore Technologies (ONT) MinION long-read sequencer to search for large insertions or gene duplication in all the evolved strains. 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. Overnight cultures were serially diluted and plated onto SP4 agarose plates containing different concentrations of antibiotics (Strep, Cip, Cm, and Ksg) to determine the MIC of each antibiotic. Plates were incubated 4-5 days at 37°C before colony counts. MIC values were defined in this study as the lowest antibiotic concentration that inhibit the growth of Syn3B (Figure S5).

Antibiotic survival assays. The schematic of the antibiotic survival assay from a stationary phase culture is shown in Figure S4. 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 72 h. Samples were taken at different time points until 72 h for the time-kill assays. 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 (Deter et al., 2019a, Levin-Reisman et al., 2017, Datla et al., 2017). Custom scripts were used to identify and count bacterial colonies (Deter et al., 2019b, Deter et al., 2019a) used to calculate CFU/ml and persister frequency. Over 200 colonies were streaked periodically into antibiotic-containing plates to test for antibiotic-resistant mutants. Also, antibiotic-treated culture was washed after 48 h, regrew to stationary phase, and exposed the culture again in the same antibiotic treatment for 48 h and plated after 24 h and 48 h to observe the difference between the first (Strep/Cip) and second death curve (Strep re-exposed/Cip re-exposed) in both antibiotic treatment. Persister assay for *E.coli* 24 h ampicillin treatment was done by similar manner as described in REF (Deter et al., 2020)

Evolution through cyclic antibiotic treatment. Stationary phase culture was exposed to 100 µg/mL streptomycin (~10× MIC), 1 µg/mL ciprofloxacin (~10× MIC), a combination of streptomycin (100 µg/mL) and ciprofloxacin (1 µg/mL) and 300 µg/mL kasugamycin (~10× MIC) antibiotic for 24 h, then antibiotic-containing medium was removed by washing twice with SP16 medium (10 min of centrifugation at 7000 g at 4°C). Finally, the culture was resuspended in 10 mL of fresh SP16 media and grown overnight at 37 °C. After every cycle of antibiotic treatment, the tolerance phenotype was observed. Finally, we isolated single colony from evolved populations from streptomycin (PS04), ciprofloxacin (PC06), combination of streptomycin and ciprofloxacin (PSC09) and kasugamycin (PK07) treatment after four, six, nine and seven cycle, respectively, and then their genomes were sequenced. Two different evolutionary lineages were used for all evolved populations.

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 (Thermo Fisher) 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 (Widdel, 2007).

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).

Supplemental References

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