Fumarate-mediated persistence of *Escherichia coli* against antibiotics

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Supplementary information

Table S1. The primers that were used for preparation of a gene knockout or operon knockout. All primers contain ~50 bp of homologous sequence for each target genomic DNA and ~20 bp of sequence for amplification of a Kan or chloramphenicol selection marker.

Knockout mutant		Sequence (5′ to 3′)
ΔSDH	FW	TGTGCCCGTAGTCCCCAGGGAATAATAAGAACAGCA
		TGTGGGCGTTATTCTGTGTAGGCTGGAGCTGCTTC
	BW	CGGCACTGGTTGCCTGATGCGACGCTTGCGCGTCTT
		ATCAGGCCTACGGTCATATGAATATCCTCCTTA
ΔFRD	FW	CTTACCCTGAAGTACGTGGCTGTGGGATAAAAACAA
		TCTGGAGGAATGTCTGTGTAGGCTGGAGCTGCTTC
	BW	CAAAACGGCCCGCCATAGGCGGGCCGGATTTACATT
		GGCGATGCGCATATGAATATCCTCCTTA
ΔCYD	FW	CTTTCTAACGGGGTTCACTCTCGGAGTCTTCATGCG
		ATGAGCAAGGAGTCTGTGTAGGCTGGAGCTGCTTC
	BW	AAGAAGCGTTCCCAGAATCCATGCGAAATACCACAT
		TTTTAGCTCCTTACCATATGAATATCCTCCTTA
ΔCYO	FW	TGCCCACACACTTTAAACGCCACCAGATCCCGTGGA
		ATTGAGGTCGTTAATGTGTAGGCTGGAGCTTC
	BW	TAATGCTAAGAAAAAAGGTGCCGTAGCACCTTTTTA
		ATAGAGAGGTTTTGCATATGAATATCCTCCTTA
ΔTOR	FW	ATTTGCTCATTAAGATCGCTTCACTAAACCATAATTC
		TACAGGGTTATTTGTGTAGGCTGGAGCTGCTTC
	BW	ATAATGCCTGATGCGACGCTAAACGCGTCTTTTCAG
		GCGCACAAACGACGCATATGAATATCCTCCTTA
ΔNAR	FW	AGCAATGTCGATTTATCAGAGGGCCGACAGGCTCCC
		ACAGGAGAAAACCGTGTGTAGGCTGGAGCTGCTTC
	BW	GCCCATAAAAAAACCCCGCCGAAGCAGGGTCTATGT
		GAACTAAAATTCGCCATATGAATATCCTCCTTA

ΔDMS	FW	AGGTGAGTCGCCGTGGTTTGGTAAAAACGACAGCG
		ATCGGCGGCCTGGCATGTGTAGGCTGGAGCTGCTTC
	BW	AACGAAGCAGAAATAAAAGAGAACCGGGGCCGAA
		ACCCCGGTTGTTATGACATATGAATATCCTCCTTA
ΔNUO	FW	ATGGTCAGCAGGATCTCAATCAGTTCCGGTGATATCC
		AACTGTGTAGGCTGGAGCTGCTTC
	BW	GCAGTATCGATTTTGTTATGTCAGATGTGGACCGCTA
		ATTCATATGAATATCCTCCTTA
Δndh	FW	CATTAATTAACAATTGGTTAATAAATTTAAGGGGGTC
		ACGTGTGTAGGCTGGAGCTGCTTC
	BW	CGGATGAGGCGTTTATGCCACATCCGCCAGTGTACG
		TCGACATCCGCCAGTGTACGTCGACATATGAATACCT
		CCTTA
ΔdctA	FW	ATAACCTTACAAGACCTGTGGTTTTACTAAAGGACA
		CCCTTGTGTAGGCTGGAGCTGCTTC
	BW	CCCGCACTCGGGGAAGGGAGTGCGGGCATAAGTGA
		TGAGACATATGAATATCCTCCTTA

Table S2. Gene knockout strains of *Escherichia coli*.

Strain	Description
BW25113	Δ(*araD-araB*)567, Δ*lacZ*4787(::*rrnB*-3), lambda-, *rph*-1, Δ(*rhaD-rhaB*)568, *hsdR*514
BW25113*ΔNUO*	*nuoEFG::Kan*
BW25113*ΔNDH*	*Ndh::Cm*
BW25113*ΔFRD*	*frdABCD*::Cm
BW25113*ΔNAR*	*narGHJI*::Kan
BW25113*ΔTOR*	*torCAD*::Kan
BW25113*ΔDMS*	*dmsABC*::Cm
BW25113*ΔCYD*	*cydAB*::Cm
BW25113*ΔCYO*	*cyoABCDE*::Kan
BW25113*ΔSDH*	*sdhCDAB*::Kan
BW25113*ΔSDHFRD*	*sdhCDAB*::Kan *frdABCD*::Cm
BW25113*ΔmenA*	*menA*::Cm
BW25113*hipA7*	G22S and D291A

Table S3. The number of bacterial species that have the genes FRD, *ampC*, and *emrB*. The first column shows gene names, the second column shows the number of bacterial species having the gene in the first column, the third column shows bacterial species having both the gene in the first column and FRD, and the fourth column shows the P value characterizing the number in the third column; a small P means that the gene in the first column and FRD tend to coexist in the same species statistically significantly. Note that the number of species that have *FRD* is 46.

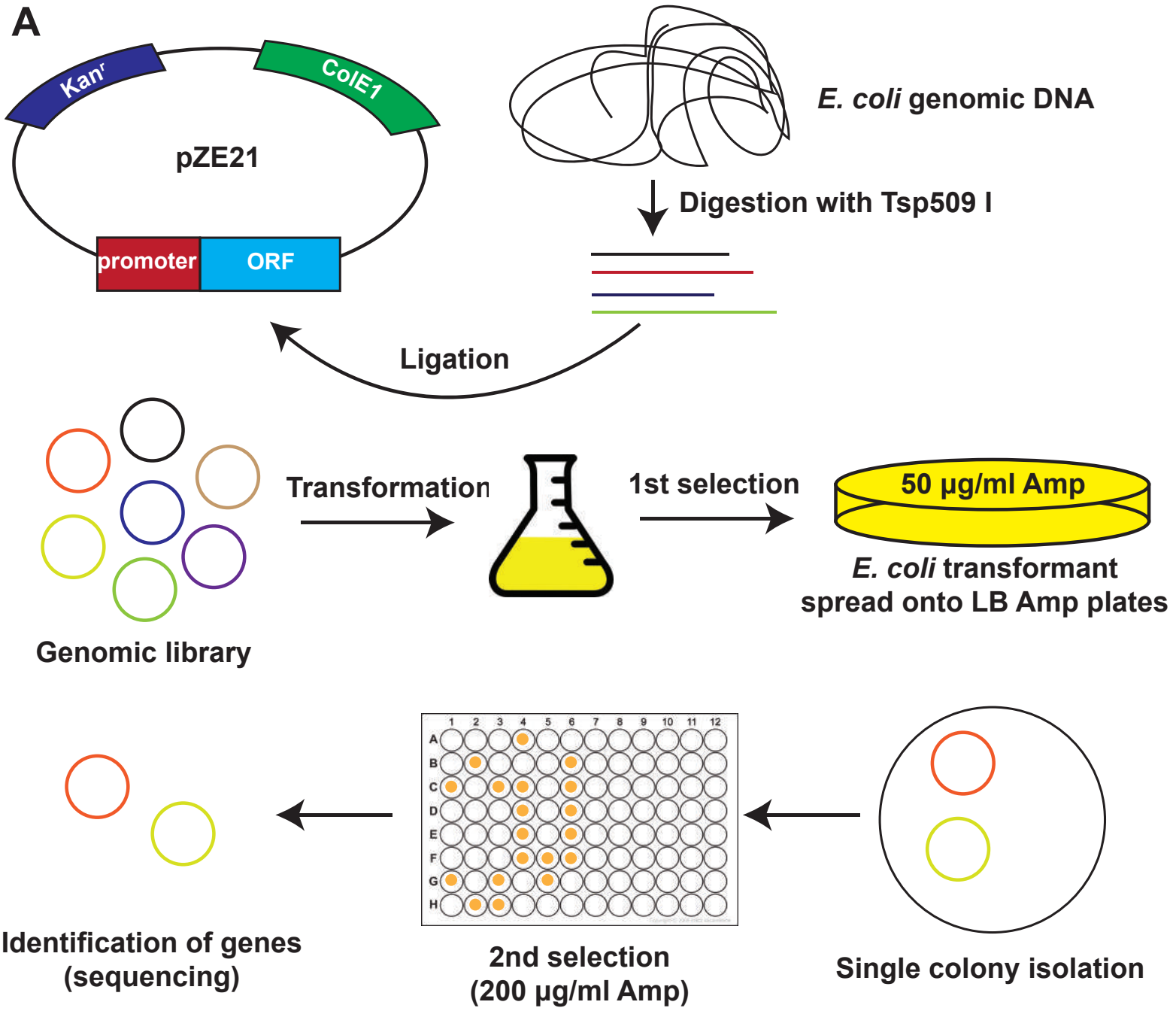
Gene	**# of species**	**# of species including both**	***P* value**
ampC	196	28	1.8×10^{-5}
emrB	129	27	2.0×10^{-10}

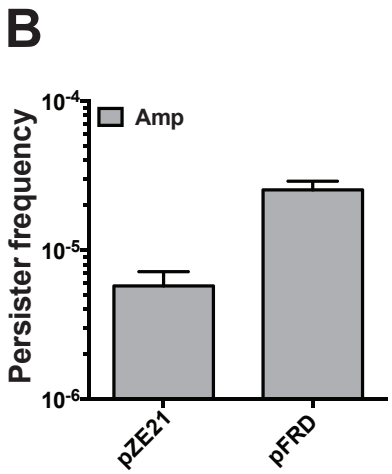
Table S4. Taxonomic clades of the 28 bacterial species that have orthologs of both FRD and ampC in their genome. [Φ]The name of a strain or substrain.

Phylum	Class	Order	Family	Genus	Species[Φ]
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Mycobacteriaceae*	*Mycobacterium*	*Mycobacterium bovis AF2122/97*
Actinobacteria	*Actinobacteria*	*Actinomycetales*	*Mycobacteriaceae*	*Mycobacterium*	*Mycobacterium tuberculosis H37Rv*
Proteobacteria	*Alphaproteobacteria*	*Rhizobiales*	*Methylobacteriaceae*	*Methylobacterium*	*Methylobacterium radiotolerans JCM 2831*
Proteobacteria	*Alphaproteobacteria*	*Rhodobacterales*	*Rhodobacteraceae*	*Rhodobacter*	*Rhodobacter sphaeroides 2.4.1*
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Comamonadaceae*	*Verminephrobacter*	*Verminephrobacter eiseniae EF01-2*
Proteobacteria	*Betaproteobacteria*	*Burkholderiales*	*Oxalobacteraceae*	*Herminiimonas*	*Herminiimonas arsenicoxydans ULPAs1*
Proteobacteria	*Betaproteobacteria*	*Neisseriales*	*Neisseriaceae*	*Chromobacterium*	*Chromobacterium violaceum ATCC 12472*
Proteobacteria	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*	*Aeromonas hydrophila subsp. hydrophila ATCC 7966*
Proteobacteria	*Gammaproteobacteria*	*Aeromonadales*	*Aeromonadaceae*	*Aeromonas*	*Aeromonas salmonicida subsp. salmonicida A449*
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Psychromonadaceae*	*Psychromonas*	*Psychromonas ingrahamii 37*
Proteobacteria	*Gammaproteobacteria*	*Alteromonadales*	*Shewanellaceae*	*Shewanella*	*Shewanella putrefaciens CN-32*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Citrobacter*	*Citrobacter koseri ATCC BAA-895*

Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter*	*Cronobacter sakazakii ATCC BAA-894*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Enterobacter*	*Enterobacter sp. 638*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Escherichia*	*Escherichia coli str. K-12 substr. MG1655*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Klebsiella*	*Klebsiella pneumoniae subsp. pneumoniae MGH 78578*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Photorhabdus*	*Photorhabdus luminescens subsp. laumondii TTO1*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Salmonella*	*Salmonella enterica subsp. enterica serovar Typhi str. CT18*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Serratia*	*Serratia proteamaculans 568*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Shigella*	*Shigella boydii Sb227*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Shigella*	*Shigella dysenteriae Sd197*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Shigella*	*Shigella flexneri 2a str. 301*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Shigella*	*Shigella sonnei Ss046*
Proteobacteria	*Gammaproteobacteria*	*Enterobacteriales*	*Enterobacteriaceae*	*Yersinia*	*Yersinia enterocolitica subsp. enterocolitica 8081*
Proteobacteria	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Photobacterium*	*Photobacterium profundum SS3*
Proteobacteria	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*	*Vibrio fischeri ES114*
Proteobacteria	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*	*Vibrio parahaemolyticus RIMD 2210633*

Proteobacteria	*Gammaproteobacteria*	*Vibrionales*	*Vibrionaceae*	*Vibrio*	*Vibrio vulnificus CMCP6*


A
Kanr
ColE1
pZE21
promoter
ORF
E. coli genomic DNA
Digestion with Tsp509 I
Ligation
Genomic library
Transformation
1st selection
50 μg/ml Amp
E. coli transformant
spread onto LB Amp plates
Identification of genes
(sequencing)
2nd selection
(200 μg/ml Amp)
Single colony isolation


B
Amp
Persister frequency
10-4
10-5
10-6
pZE21
pFRD

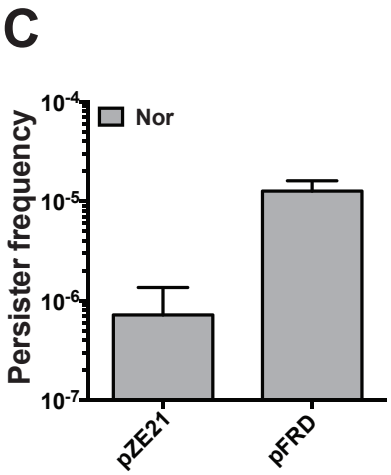

C
Nor
Persister frequency
10-4
10-5
10-6
10-7
pZE21
pFRD

Fig. S1. Screening of a genomic library for persistence-enhancing genes and operons. (A) *Escherichia coli* K12 genomic fragments (2–6 kbp) were inserted into the plasmid pZE21 according to the procedure described in Materials and Methods. The plasmids were introduced into *E. coli* DH5α cells, and the transformants were selected with Kan. The *E. coli* DH5α strain (an equivalent of 10^7 cells) that was transformed with the genomic library was spread on a Luria-Bertani (LB) agar plate containing Amp (50 μg/mL). After cultivation at 37 °C for three days, 48 colonies were randomly selected. Each colony was then exposed to 200 μg/mL Amp in LB broth for 16 h. Nineteen transformants survived this harsh treatment and gave rise to colonies when subsequently spread on LB agar plates without Amp. All plasmids were isolated and sequenced to identify the genes that improved the antibiotic tolerance. (B, C) Overexpression of *FRD* by means of the pFRD vector increases persister frequency toward antibiotics at exponentail phase.

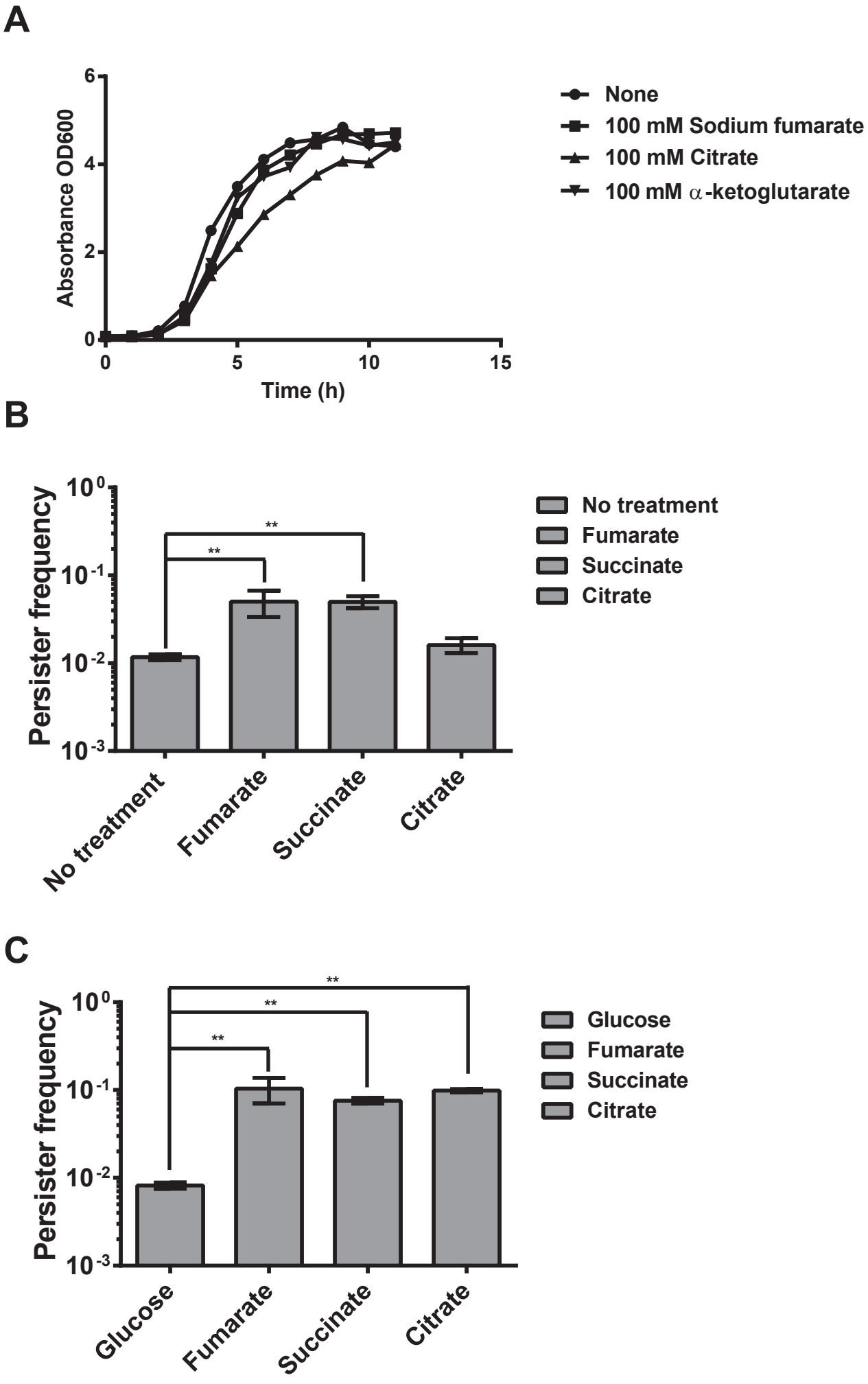
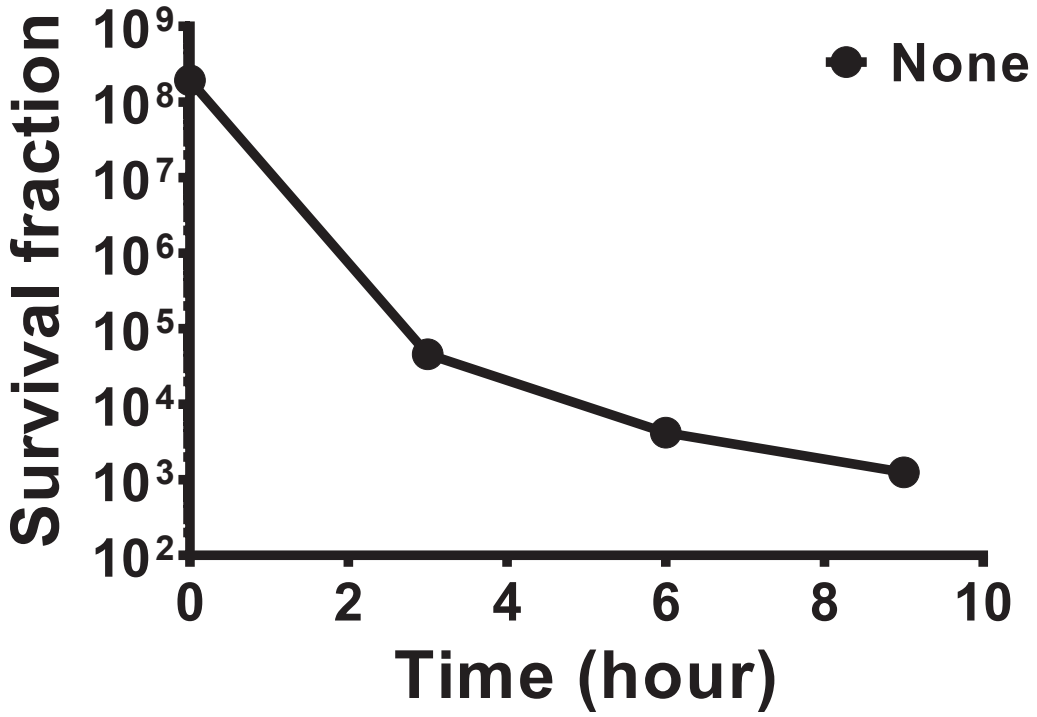

A
Absorbance OD600
Time (h)
None
100 mM Sodium fumarate
100 mM Citrate
100 mM α-ketoglutarate
B
Persister frequency
No treatment
Fumarate
Succinate
Citrate
**
C
Glucose
Fumarate
Succinate
Citrate

Fig. S2. The effect of TCA intermediates pre-exposure on persister formation. (A) Cell growth profile in the presence of various TCA intermediates in LB. When persister frequency was measured cells were harvested and treated with antibiotics in fresh LB broth after pre-exposure to TCA intermediates in LB. Cell growth was not severely inhibited by 100 mM TCA intermediates in LB broth during this pre-exposure stage. (B) When the cells pre-exposed to TCA intermediates (100 mM) until exponential phase, harvested and were resuspended in fresh M9 broth containing 5 μg/mL Nor, persister frequency was elevated by fumarate or succinate but was not changed by citrate. (C) When TCA intermediates (6.75 mM) were present together with 5 μg/mL Nor and were used as sole carbon source in M9 broth, persister frequency was similarly affected by different intermediates, emphasizing that pre-accumulated fumarate inside cells plays role in persister formation.

A


Survival fraction
10^9
10^8
10^7
10^6
10^5
10^4
10^3
10^2
None
0
2
4
6
8
10
Time (hour)

B

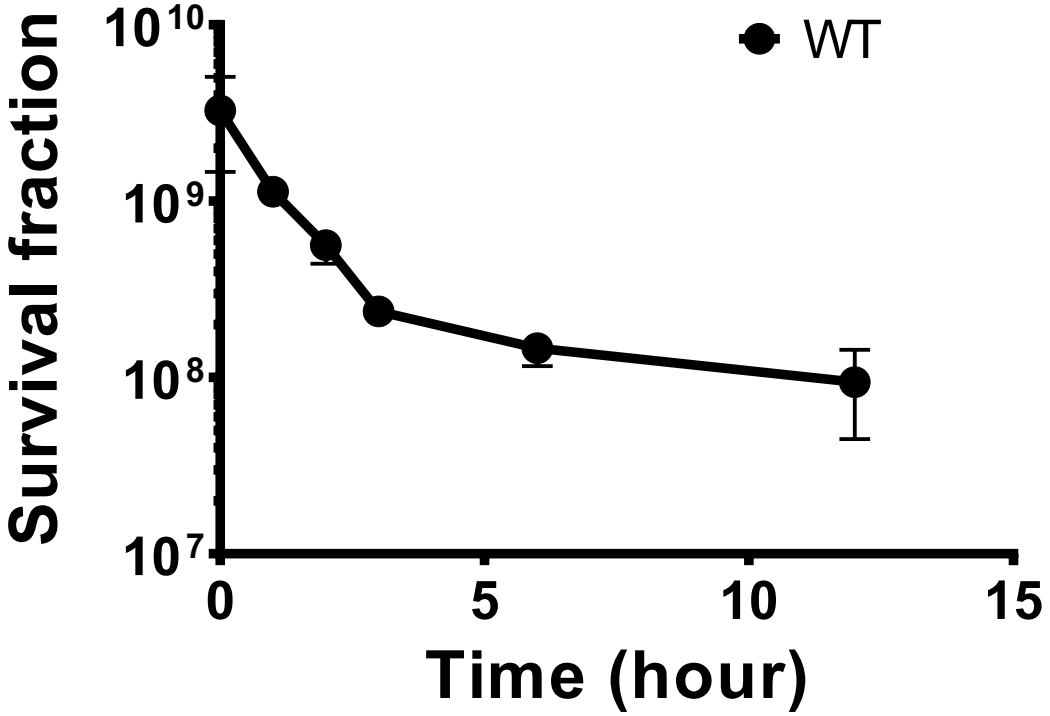

Survival fraction
10^10
10^9
10^8
10^7
WT
0
5
10
15
Time (hour)

Fig. S3. Nor-induced cell death kinetics. (A) Biphasic death kinetics of cells at exponential phase. Cell death rate was reduced after 3 h of Nor treatment. (B) Biphasic death kinetics of cells at at stationary phase.

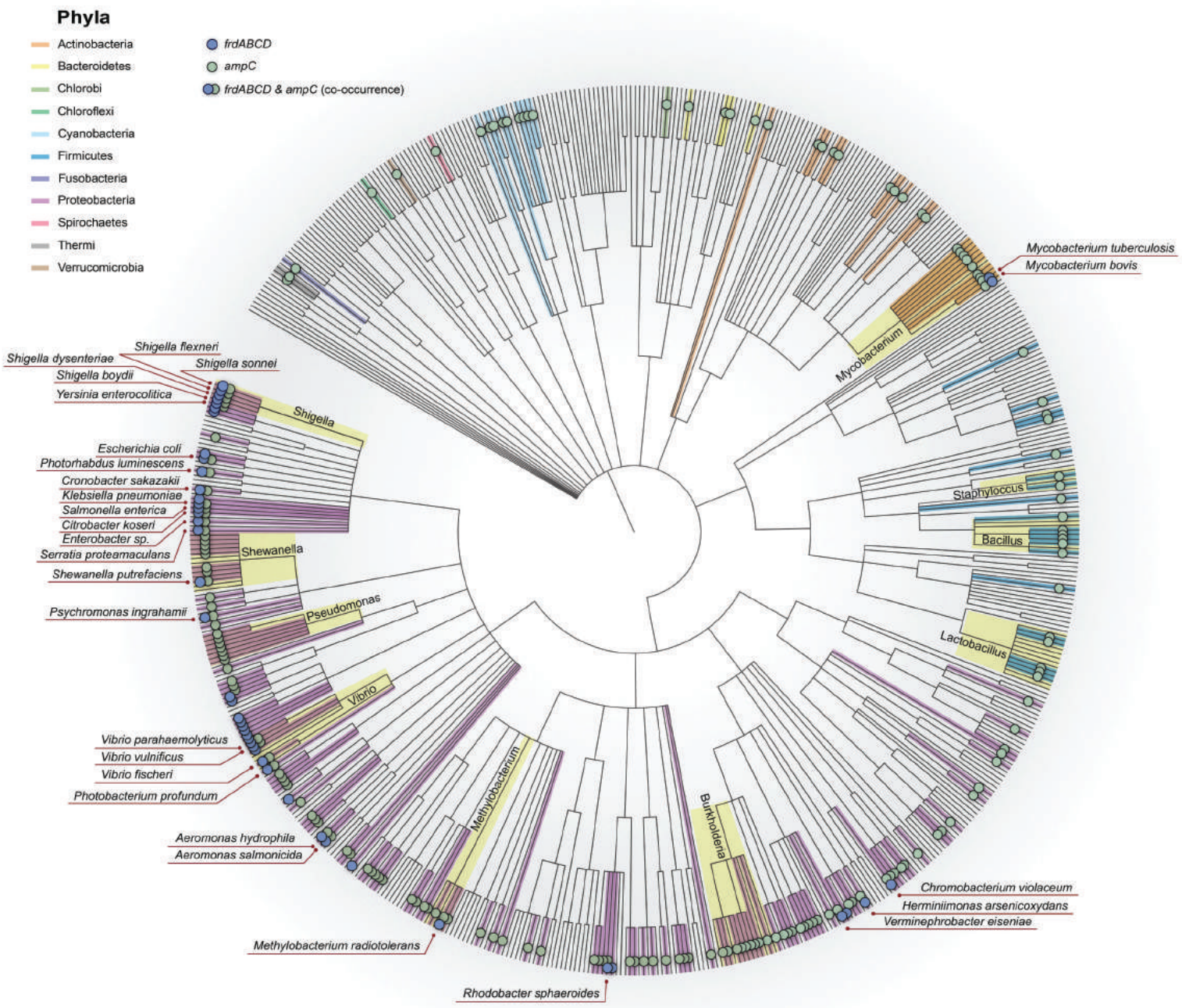

Phyla
Actinobacteria
Bacteroidetes
Chlorobi
Chloroflexi
Cyanobacteria
Firmicutes
Fusobacteria
Proteobacteria
Spirochaetes
Thermi
Verrucomicrobia
frdABCD
ampC
frdABCD & ampC (co-occurrence)
Mycobacterium tuberculosis
Mycobacterium bovis
Mycobacterium
Shigella flexneri
Shigella dysenteriae
Shigella sonnei
Shigella boydii
Yersinia enterocolitica
Shigella
Escherichia coli
Photorhabdus luminescens
Cronobacter sakazakii
Klebsiella pneumoniae
Salmonella enterica
Citrobacter koseri
Enterobacter sp.
Serratia proteamaculans
Shewanella
Shewanella putrefaciens
Psychromonas ingrahamii
Pseudomonas
Vibrio
Vibrio parahaemolyticus
Vibrio vulnificus
Vibrio fischeri
Photobacterium profundum
Aeromonas hydrophila
Aeromonas salmonicida
Methylobacterium
Methylobacterium radiotolerans
Rhodobacter sphaeroides
Burkholderia
Chromobacterium violaceum
Herminiimonas arsenicoxydans
Verminephrobacter eiseniae
Staphylococcus
Bacillus
Lactobacillus

Fig. S4. Taxonomic Signatures of *FRD* Are Related to Antibiotic Tolerance. To confirm the role of FRD in the persistence, we performed the identification of taxonomic and genomic signatures of FRD in bacterial genomes. First, we found that orthologs of *FRD* and *ampC* tended to be present in the genome of the same organism across numerous bacterial species: much more frequently than what can be expected by chance (P = 1.8 × 10–5; Table S3). The bacteria carrying both orthologs even belonged to lineages of two phyla: Actinobacteria and roteobacteria (Fig. S4 and Table S4), meaning that this co-occurrence was not limited to closely related taxa only. Similarly, we observed significant co-occurrence of another antibiotic-resistant gene (*emrB*) with *FRD* (P = 2.0 × 10–10; Table S1). Because genes with favored co-occurrence across organisms are likely to have related biological functions (1, 2), such co-occurrence of *FRD* and antibiotic-resistance genes supported our experimental findings about the role of *FRD* in antibiotic tolerance. Next, we compared the genomic locations of *FRD* and *ampC*. We found that not only does *E. coli* have FRD in close proximity to *ampC* in its genome (Fig. 1A) but so do other bacterial species of the same *Enterobacteriaceae* family, such as *Enterobacter sp. 638* and *Shigella boydii* (Fig. S5). Taken together with our experimental findings, these taxonomic and genomic signatures were suggestive of a putative role of the *FRD* complex in antibiotic tolerance.

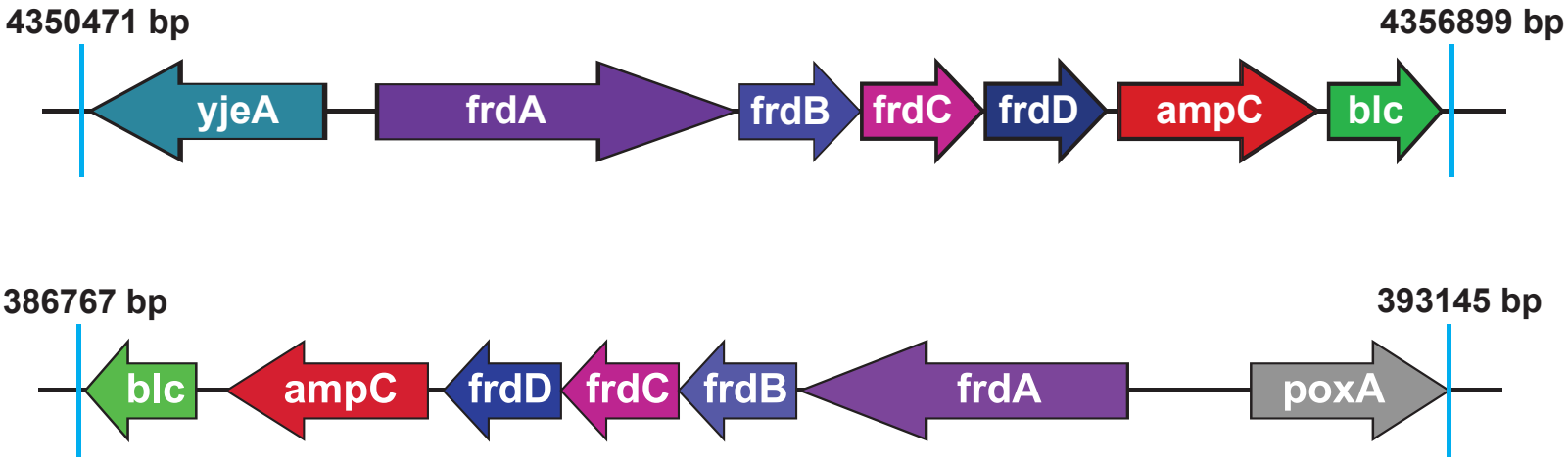

4350471 bp
4356899 bp
yjeA
frdA
frdB
frdC
frdD
ampC
blc
386767 bp
393145 bp
blc
ampC
frdD
frdC
frdB
frdA
poxA

Fig. S5. Schematic representation of the *FRD* and *ampC* gene cluster in (A) *Enterobacter sp. 638* and (B) *Shigella boydii Sb227*. Each arrow indicates the direction of transcription and relative size of the open reading frame. The sequence locations were obtained from the National Center for Biotechnology Information (NCBI) Genome Browser. In total, the following bacterial species have *FRD* proximal to *ampC* in their genome: *Escherichia coli, Enterobacter sp. 638, S. boydii, S. dysenteriae, S. flexneri,* and *S. sonnei*.

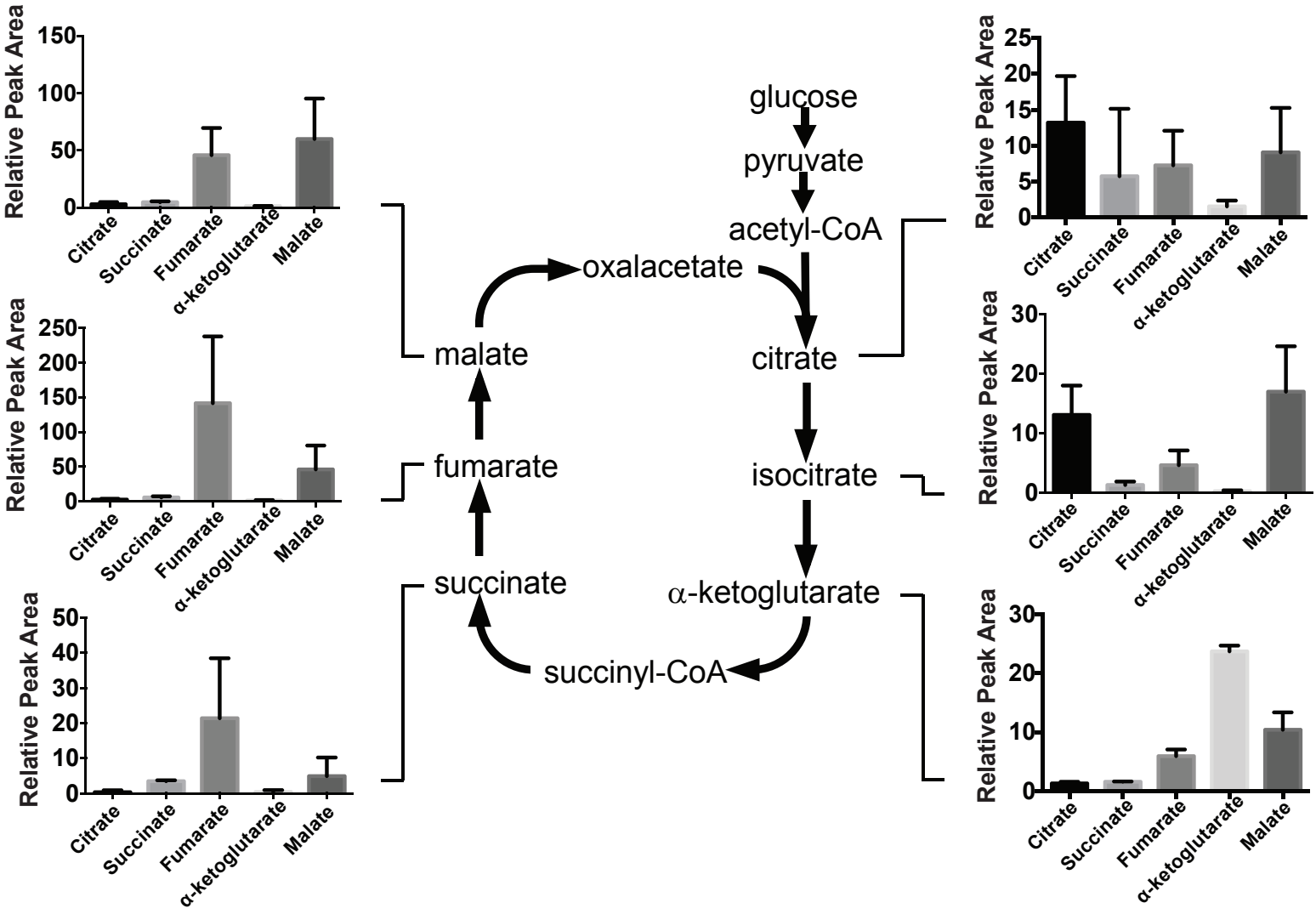
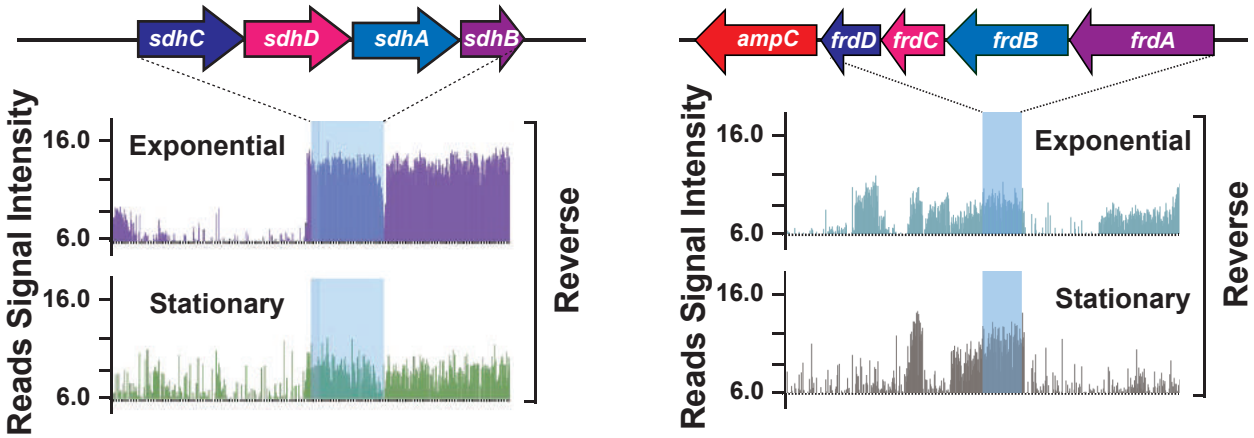

Relative Peak Area
150
100
50
0
Citrate
Succinate
Fumarate
α-ketoglutarate
Malate
Relative Peak Area
250
200
150
100
50
0
Citrate
Succinate
Fumarate
α-ketoglutarate
Malate
Relative Peak Area
50
40
30
20
10
0
Citrate
Succinate
Fumarate
α-ketoglutarate
Malate
malate
fumarate
succinate
oxalacetate
glucose
pyruvate
acetyl-CoA
citrate
isocitrate
α-ketoglutarate
succinyl-CoA
Relative Peak Area
25
20
15
10
5
0
Citrate
Succinate
Fumarate
α-ketoglutarate
Malate
Relative Peak Area
30
20
10
0
Citrate
Succinate
Fumarate
α-ketoglutarate
Malate
Relative Peak Area
30
20
10
0
Citrate
Succinate
Fumarate
α-ketoglutarate
Malate

Fig. S6. Changes in the overall composition of the tricarboxylic acid (TCA) cycle after supplementation of the culture medium with each intermediate. After addition of a TCA intermediate to the culture medium until exponentail phase, intracellular metabolites were extracted from the bacterial cells and were subjected to gas chromatography with time-of-flight mass spectrometry (see Materials and Methods).

A


sdhC
sdhD
sdhA
sdhB
Reads Signal Intensity
16.0
6.0
Exponential
Stationary
Reverse
ampC
frdD
frdC
frdB
frdA
16.0
6.0
Exponential
Stationary
Reverse

B

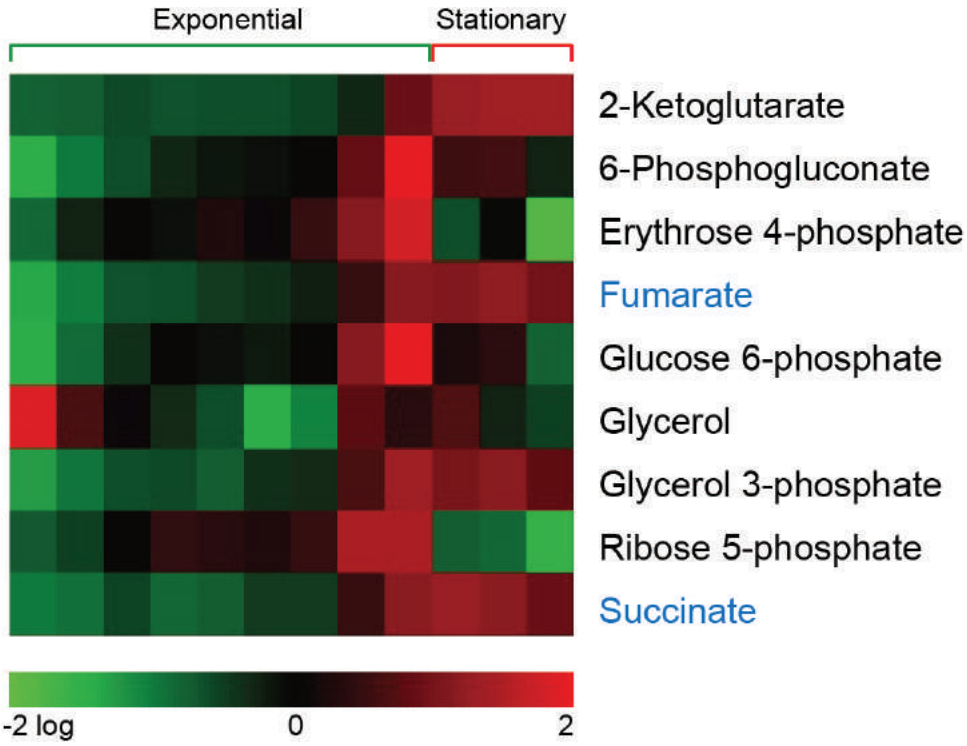

Exponential
Stationary
2-Ketoglutarate
6-Phosphogluconate
Erythrose 4-phosphate
Fumarate
Glucose 6-phosphate
Glycerol
Glycerol 3-phosphate
Ribose 5-phosphate
Succinate
-2 log
0
2

Fig. S7. Analysis of deposited data regarding the fumarate metabolism in *E. coli*. (A) *FRD* expression is enhanced in the stationary phase, and *SDH* is highly expressed in exponential phase. For growth stage-specific transcription of *Escherichia coli FRD* and *SDH* operons during growth, we used Gene Expression Omnibus (GEO) data on *E. coli* K-12 MG1655 grown in the M9 glucose minimal medium (accession No. GSE15534). The source data were obtained from (3). The expression levels were visualized using SignalMap (Roche NimbleGenm, Basel, Switzerland). (B) Changes in metabolites of *Escherichia coli* K-12 MG1655 during growth in the M9 glucose minimal medium under aerobic conditions. The source data were obtained from (4).

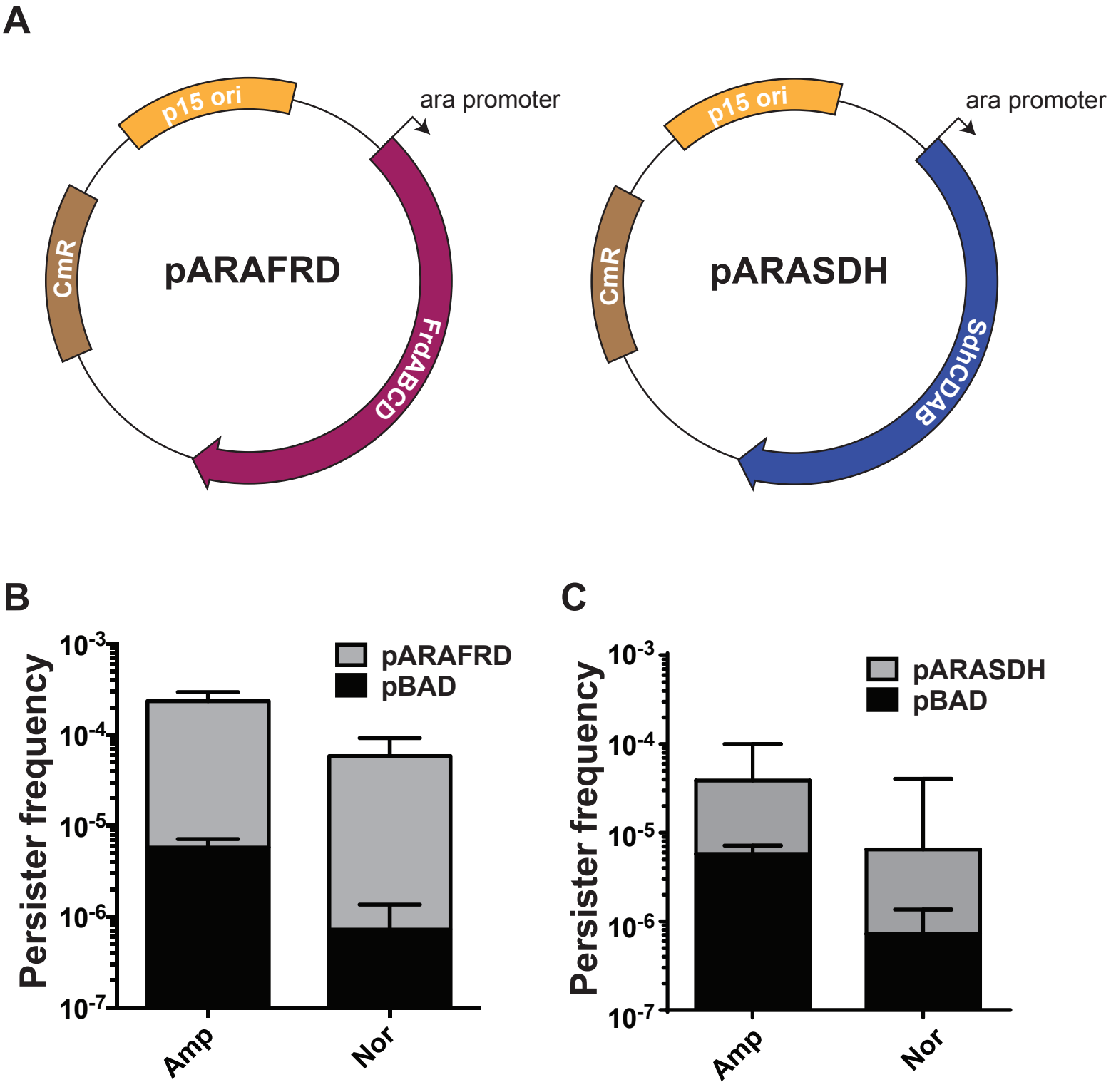
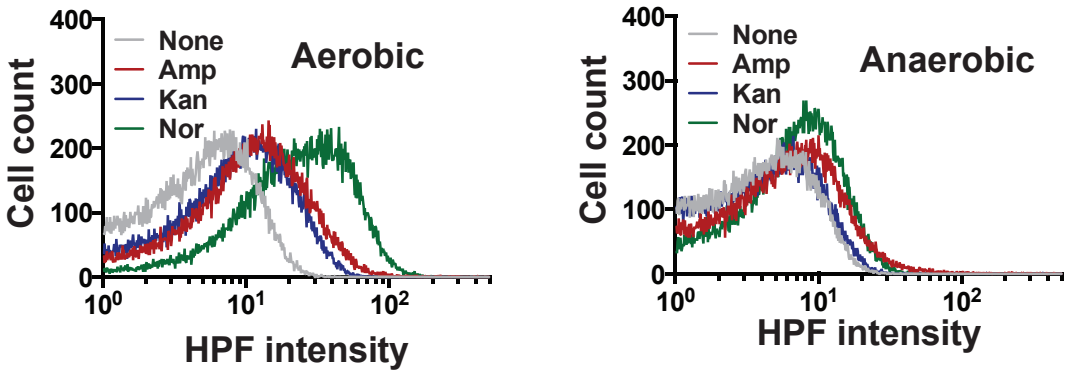

A
p15 ori
ara promoter
CmR
pARAFRD
FrdABCD
p15 ori
ara promoter
CmR
pARASDH
sdhCDAB
B
Persister frequency
10-3
10-4
10-5
10-6
10-7
pARAFRD
pBAD
Amp
Nor
C
Persister frequency
10-3
10-4
10-5
10-6
10-7
pARASDH
pBAD
Amp
Nor

Fig. S8. The effects of overexpression of *SDH* or *FRD* on persister frequency. (A) Maps of the plasmids containing arabinose-inducible fumarate reductase or succinate dehydrogenase. (B) Overexpression of *SDH* by the arabinose-inducible promoter increases the persister frequency. (C) Overexpression of *FRD* by the arabinose-inducible promoter increases the persister frequency. Cell at exponentail phase was used for this experiments.

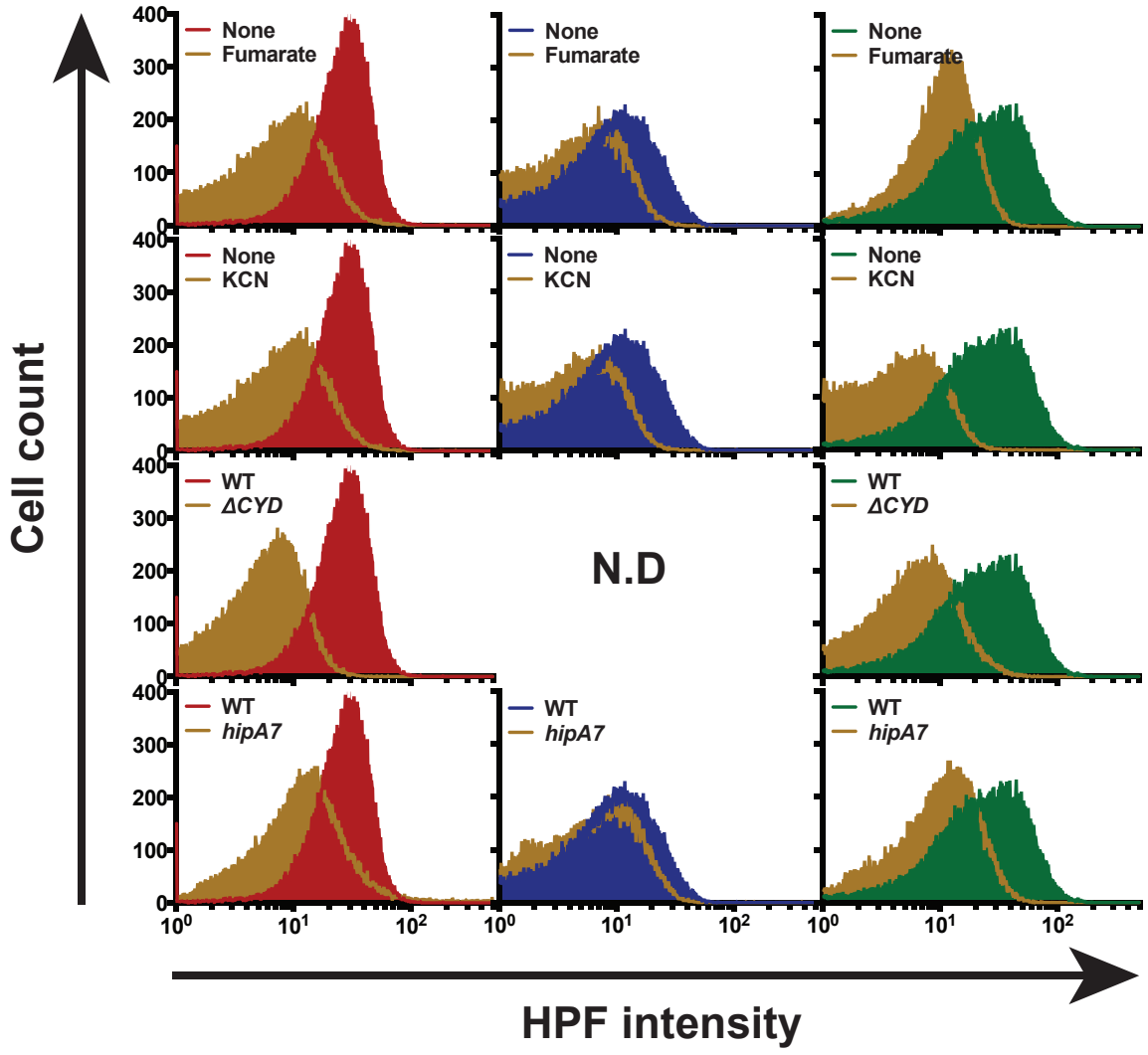
A
CYO
CYD
NAR
FRD
DMS
TOR
Terminal reductase and oxidase
O2 + 4 H+
2 H2O
O2 + 4 H+
2 H2O
NO3- + 2 H+
NO2- + H2O
Fumarate + 2 H+
Succinate
DMSO + 2 H+
TMAO + 2 H+
TMA + H2O
DMS + H2O
B
Relative persister frequency
10^3
10^2
10^1
10^0
10^-1
None
Fumarate
Nitrate
DMSO
TMAO
C
Persister frequency
10^-5
10^-6
10^-7
10^-8
WT
ΔNAR
ΔTOR
ΔDMS

Fig. S9. The effects of terminal oxidoreductases and alternative electron acceptors on *E. coli* persister frequency. (A) The reaction scheme of the electron transport chain that is mediated by each terminal oxidoreductase. NAR, nitrate reductase operon; DMS, dimethyl sulfoxide reductase operon; TOR, trimethylamine N-oxide (TMAO) reductase operon. (B) The effects of alternative electron acceptors on the persister frequency under aerobic condition. Only fumarate increased the persister frequency of stationary-phase cells. (C) Terminal reductase operons mediating anaerobic respiration were deleted from the chromosome of *Escherichia coli*, and the persister frequency was measured using each knockout mutant. None of the mutations affected persister frequency under aerobic condition as strongly as the deletion of SDH did. Both experiments (B, C) indicated that fumarate is the major alternative electron acceptor that allowed the bacterial cells to tolerate antibiotics. Cell at exponential phase was used for this experiments.

A


Aerobic
None
Amp
Kan
Nor
Cell count
HPF intensity
Anaerobic
None
Amp
Kan
Nor
Cell count
HPF intensity

B


+Amp
+Kan
+Nor
Cell count
None
Fumarate
None
KCN
WT
ΔCYD
WT
hipA7
N.D
HPF intensity

C

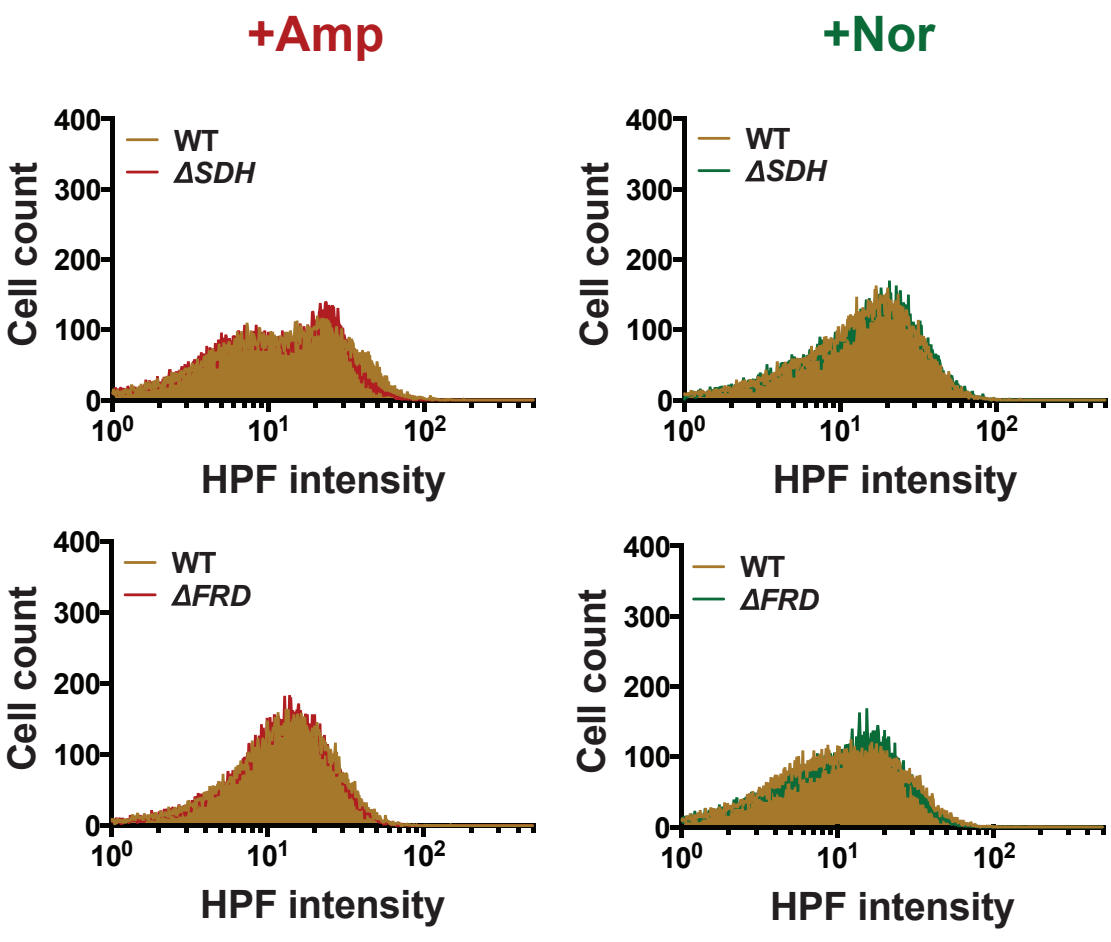

+Amp
+Nor
Cell count
WT
ΔSDH
WT
ΔFRD
HPF intensity

Fig. S10. FACS histograms measured at different conditions. (A) As was first demonstrated by Kohanski et al. (5), low concentration of antibiotics (10 μg/mL Amp, 10 μg/mL Kan and 0.5 μg/mL Nor) induced the increase of HPF intensity. Cells under anaerobic conditions did not produce ·OH. (B) Supplementation of KCN or Δ*CYD*, that blocks aerobic respiration, reduced ·OH generation. Pre-exposure of fumarate and the *hipA7* mutation reduced ·OH generation even under aerobic conditions. (C)Though the deletion of *SDH* reduced the persister frequency due to lowered intracellular fumarate concentration, the knock-out mutant did not exhibit increased ·OH generation. Cell at exponentail phase was used for this experiments.

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