Supplementary Materials and Methods

In vitro evolution of *E. coli* strain BW25113 and whole-genome sequencing characterization

E. coli strain BW25113 was gradually exposed over five days to increasing concentrations of ciprofloxacin (from 1/4xMIC to 4xMIC) in serial subcultures. Stable MIC increases were confirmed for several mutants obtained at 4xMIC and their mutations were characterized by whole genome sequencing (WGS). Genomic DNA from wild-type and evolved mutants were extracted and sequenced using MiSeq (Illumina, San Diego, CA, USA), generating 300 bp paired-end reads. The library was prepared using the Nextera XT DNA library preparation kit (Illumina), which involves DNA fragmentation, adaptor ligation and amplification. Raw reads were quality filtered and assembled into contigs using the CLC genomics workbench 9.5.2. (CLC bio, QIAGEN) and the default settings of the software. An average coverage of 50× was observed. Contigs were annotated using RAST 2.0 (http://rast.nmpdr.org/rast.cgi). SNP analysis and mutation/variant detection was carried out in a CLC genomics workbench 9.5.2 using *E. coli* BW25113 (Genbank accession: CP009273) as the reference. BLASTn/p® (National Center for Biotechnology Information, Bethesda, MD, USA) was used to compare sequences. After this analysis, it was confirmed that the *E. coli* BW15 strain harboured four changes, including D87G substitution in *gyrA* gene, as molecular mechanisms of ciprofloxacin resistance (see Table S1).

Monitoring the induction of the SOS system

To validate the genetic constructions aimed at inactivation of the SOS response, the low-copy plasmid, pMSrecA-gfp, a GFP reporter-promoter fusion, (1) was introduced into all mutants in our collection by electroporation. To show recA promoter activity (SOS induction) after the addition of ciprofloxacin, the disc diffusion method was performed, readings were made after 24 h of incubation at 37ºC using the Safe Imager™ 2.0 (Invitrogen, Madrid, Spain).

Antioxidant Pretreatment

Stock solution of L-Ascorbic acid at 1 M (Sigma-Aldrich, Madrid, Spain) was prepared in dH_2O and titrated to pH 6.8 with 1 N NaOH (Panreac AppliChem, Barcelona, Spain). To test the effects of pre-treatment with L-Ascorbic acid, overnight cultures of studied strains were inoculated in LB and grown at 37°C and 180 rpm in an incubator shaker with light-protection. An initial inoculum of 10^6 CFU/mL was used in all experiments. Cultures were pretreated with 50mM L-Ascorbic acid pH 6.8 for 10 min. After antioxidant pre-treatment, ciprofloxacin concentrations of 1xMIC for the Δ*recA* strain were used. Growth in drug-free and L-Ascorbic acid-free LB were evaluated in parallel as controls. Viable cells were determined at 4 hours by colony counting. At least three biological replicas were measured for each condition.

ROS detection using DCFH-DA

In order to determine whether changes in susceptibility, associated with active or inactive *recA* gene and oxidative detoxification systems, correlated with ROS formation, the radical-sensitive reporter dye, fluorescent 2',7'–dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich), was used to examine the levels of oxidizing radicals as previously described using an Infinite 200 PRO plate reader. Bacterial cells were treated with ciprofloxacin concentration of 1xMIC (relative to the Δ*recA* mutant). At least four biological replicas per condition were measured in at least two independent assays.

Supplementary Figures.

Table S1. Genotypes, FIC and fluoroquinolones (CIP: Ciprofloxacin; LVX: Levofloxacin; MFX: Moxifloxacin; NFX: Norfloxacin; OFX: Ofloxacin; NAL: Nalidixic acid) susceptibility (by gradient strips) of isogenic strains.

Table S2. Oligonucleotides, phages and plasmids used in this study.

Figure S1. Monitoring the SOS response in engineered *E. coli* BW25113 with *recA* gene inactivation and detoxification system inactivation for all combinations used in this study. Ciprofloxacin induces DNA damage leading to activation of the SOS response. Ciprofloxacin-induced GFP expression from the pMSrecA-gfp vector was used to display *recA* promoter activity (SOS induction) after the addition of a fluoroquinolone (ciprofloxacin). Pictures of the disc diffusion method with ciprofloxacin (5 µg) at 24 hours are shown. Green fluorescence indicates induction of SOS response.

Figure S2. Growth curves for *E. coli* BW25113 and all genetic combinations for groups with ciprofloxacin concentrations of 0.0005 (A), 0.001 (B) and 0.002 mg/L (C, D, E, F and G) during 24 hours. Data are represented as the mean of at least three independent measurements. Growth curves of all constructions was similar in non-antibiotic control (data not shown).

Figure S3. Viable bacterial counts of *E. coli* BW25113 with *recA* gene and detoxification system inactivation (*katG*, *sodA* and *ahpC*) combinations by groups in time-kill assays at ciprofloxacin (CIP) concentrations of 1xMIC *E. coli* BW25113 (0.015 mg/L) during 24 h. Data are represented as the means of at least three independent measurements. Time-kill curves of all constructions was similar in non-antibiotic control (data not shown). Standard deviations are shown (values not represented are inside the symbol). CIP, ciprofloxacin.

Figure S4. Bactericidal activity of quinolones is diminished with L-Ascorbic acid pH 6.8 pretreatment even if *recA* gene and detoxification systems, in combination, are suppressed. Viable bacterial counts of *E. coli* Δ*recA*, *E. coli* Δ*katG*/Δ*recA*, *E. coli* Δ*sodA*/Δ*recA* and *E. coli* Δ*ahpC*/Δ*recA* in time-kill assays at ciprofloxacin (CIP) concentrations of 1xMIC Δ*recA* (0.004 mg/L) with or without pretreatment with L-Ascorbic acid (50mM) at 4 hours. Data are represented as the means of at least three independent measurements. *E. coli* BW25113 was not included due to the antibiotic concentration used was under its MIC, so that its behaviour was similar to non-antibiotic control. Error bars represent standard deviations. Significant P values are noted (*: compared to strain without treatment with $p<0.001$; #: compared to strain with ciprofloxacin treatment with $p<0.01$).

Figure S5. Combination of *recA* gene inactivation and detoxification system suppression inactivation increases ROS production in isogenic *E. coli* strains under selective quinolone concentrations. ROS measurement for *E. coli* and all genetic combinations with ciprofloxacin concentrations of 1xMIC of Δ*recA* (0.004 mg/L) at 24 h. Data were normalized to a no-dye control (background fluorescence) and OD595nm (bacterial density) and are represented as the mean of at least four independent measurements. ROS production of all constructions was similar in non-antibiotic control (data not shown). Error bars represent standard deviations. Significant P values are noted (*, <0.05; ***, <0.001).

Figure S6. Growth curves for *E. coli* BW25113 with *recA* gene and *katG* detoxification system inactivation alone or in combinations with ciprofloxacin concentrations from 8xMIC to 1/256xMIC of *E. coli* BW25113 during 24 hours. Data are represented as the mean of at least three independent measurements. Growth curves of all constructions were also performed without antibiotic in parallel (Untreated).

Figure S7. Viable bacterial counts of *E. coli* BW25113 with *recA* gene and *katG* detoxification system inactivation alone or in combinations in time-kill assays at ciprofloxacin (CIP) concentrations of 4xMIC (0.06 mg/L), 1xMIC (0.015 mg/L), 1/2xMIC (0.008 mg/L) and 1/4xMIC (0.004 mg/L) of *E. coli* BW25113 during 24 h. Data are represented as the means of at least three independent measurements. Time-kill

curves of all constructions were also performed without antibiotic in parallel (Untreated). Standard deviations are shown. CIP, ciprofloxacin.

Table S1. Genotypes, FIC and fluoroquinolones (CIP: Ciprofloxacin; LVX: Levofloxacin; MFX: Moxifloxacin; NFX: Norfloxacin; OFX: Ofloxacin; NAL: Nalidixic acid) susceptibility (by gradient strips) of isogenic strains.

Strain[a]	Mechanism of quinolone resistance[b]					SOS/ROS system	MIC (Fold)[c]							FIC (CIP)[g]	Source or reference
	gyrA	*gyrB*	*parC*	*parE*	*marR*		CIP[d]	CIP[e]	LVX	MFX	NFX	OFX	NAL		
BW25113	-	-	-	-	-	WT[f]	0.015	0.06	0.047	0.047	0.094	0.064	6	-	-
BW25113katG	-	-	-	-	-	Δ*katG*	0.015	0.06	0.032 (1.5)	0.047	0.094	0.047 (1.5)	6	-	(2)
BW25113katE	-	-	-	-	-	Δ*katE*	0.015	0.06	0.032 (1.5)	0.064 (0.7)	0.125 (0.7)	0.064	4 (1.5)	-	(2)
BW25113sodA	-	-	-	-	-	Δ*sodA*	0.015	0.06	0.032 (1.5)	0.047	0.094	0.032 (2)	12 (0.5)	-	(2)
BW25113sodB	-	-	-	-	-	Δ*sodB*	0.015	0.06	0.032 (1.5)	0.047	0.094	0.047 (1.5)	6	-	(2)
BW25113ahpC	-	-	-	-	-	Δ*ahpC*	0.015	0.06	0.047	0.047	0.064 (1.5)	0.047 (1.5)	4 (1.5)	-	(2)
BW25113oxyR	-	-	-	-	-	Δ*oxyR*	0.015	0.06	0.047	0.047	0.125 (0.7)	0.094 (0.7)	6	-	(2)

BW25113rpoS	-	-	-	-	-	Δ*rpoS*	0.015	0.06	0.032 (1.5)	0.047	0.125 (0.7)	0.047 (1.5)	6	-	(2)
BW25113katG/katE	-	-	-	-	-	Δ*katG*Δ*katE*	0.015	0.06	0.047	0.047	0.094	0.047 (1.5)	4 (1.5)	-	This study
BW25113sodB/sodA	-	-	-	-	-	Δ*sodB*Δ*sodA*	0.015	0.06	0.047	0.094 (0.5)	0.125 (0.7)	0.047 (1.5)	4 (1.5)	-	This study
BW25113recA	-	-	-	-	-	Δ*recA*	0.004 (4)	0.008 (7.5)	0.008 (6)	0.004 (12)	0.023 (4)	0.016 (4)	1 (6)	-	(2)
BW25113katG/recA	-	-	-	-	-	Δ*katG*Δ*recA*	0.001 (16)	0.008 (7.5)	0.006 (8)	0.008 (6)	0.023 (4)	0.008 (8)	2 (3)	0.32	This study
BW25113katE/recA	-	-	-	-	-	Δ*katE*Δ*recA*	0.002 (8)	0.008 (7.5)	0.008 (6)	0.012 (4)	0.047 (2)	0.012 (5)	3 (2)	0.63	This study
BW25113sodA/recA	-	-	-	-	-	Δ*sodA*Δ*recA*	0.001 (16)	0.008 (7.5)	0.008 (6)	0.008 (6)	0.032 (3)	0.008 (8)	1.5 (4)	0.32	This study
BW25113sodB/recA	-	-	-	-	-	ΔsodBΔ*recA*	0.001 (16)	0.008 (7.5)	0.006 (8)	0.008 (6)	0.032 (3)	0.012 (5)	2 (3)	0.32	This study
BW25113ahpC/recA	-	-	-	-	-	Δ*ahpC*Δ*recA*	0.001 (16)	0.008 (7.5)	0.006 (8)	0.006 (8)	0.016 (6)	0.008 (8)	1.5 (4)	0.32	This study
BW25113oxyR/recA	-	-	-	-	-	Δ*oxyR*Δ*recA*	0.0005 (32)	0.008 (7.5)	0.008 (6)	0.008 (6)	0.032 (3)	0.012 (5)	2 (3)	0.16	This study
BW25113rpoS/recA	-	-	-	-	-	Δ*rpoS*Δ*recA*	0.002 (8)	0.008 (7.5)	0.008 (6)	0.008 (6)	0.032 (3)	0.008 (8)	2 (3)	0.63	This study
BW25113katG/katE/recA	-	-	-	-	-	Δ*katG*Δ*katE*Δ*recA*	0.001 (16)	0.008 (7.5)	0.008 (6)	0.008 (6)	0.023 (4)	0.006 (11)	2 (3)	0.32	This study

BW15	D87G	E465D	-	K390N	G414_C430*del*	-	2	-	6	16	12	12	>256	-	This study
BW15katG	D87G	E465D	-	K390N	G414_C430*del*	Δ*katG*	2	-	6	16	16 (0.7)	16 (0.7)	>256	-	This study
BW15sodA	D87G	E465D	-	K390N	G414_C430*del*	Δ*sodA*	2	-	6	16	16 (0.7)	16 (0.7)	>256	-	This study
BW15recA	D87G	E465D	-	K390N	G414_C430*del*	Δ*recA*	0.5 (4)	-	3 (2)	3 (5.5)	6 (2)	6 (2)	>256	-	This study
BW15katG/recA	D87G	E465D	-	K390N	G414_C430*del*	Δ*katG*Δ*recA*	0.5 (4)	-	3 (2)	3 (5.5)	6 (2)	6 (2)	>256	-	This study
BW15sodA/recA	D87G	E465D	-	K390N	G414_C430*del*	Δ*sodA*Δ*recA*	0.5 (4)	-	3 (2)	3 (5.5)	6 (2)	6 (2)	>256	-	This study

[a] Genotype. Strains are isogenic to *E. coli* BW25113 and carry only chromosomal modifications, SOS dysfunction [*recA* deletion] and/or detoxification system dysfuction. Resistance-associated mutations located in the GyrA and ParC proteins have been defined as resistance mechanisms that alter the target site.

[b] Aminoacidic changes [*gyrA*, *gyrB*, *parC* and *parE* genes] and nucleotidic changes [*marR*].

[c] MIC (mg/L) by gradient strips (Number of fold reduction in MIC of each quinolone compared to wild-type strain for each isogenic subgroup).

[d]MIC to Ciprofloxacin in aerobiosis.

[e]MIC to Ciprofloxacin in anaerobiosis.

[f]Wild-type

[g]FIC means Fractional Inhibitory Concentration

Table S2. Oligonucleotides, phages and plasmids used in this study.

Primer or plasmid	Sequence	Use in this study	Source or reference
Inactivation of chromosomal genes			
Pre-H1-recA	5’ -TCGTCAGGCTACTGCGTATGCAT-3’	*recA* inactivation testing	This study
Post-H2-recA	5’ -GTACCGCACGATCCAACAGGCGA-3’	*recA* inactivation testing	This study
Pre-katG	5’ CGGACATAATCAAAAAAGCTTA 3’	*katG* inactivation testing	This study
Post-katG	5’ GAGCACAACCAGGCCACTGATTG 3’	*katG* inactivation testing	This study
Pre-katE	5’ CCCTGTACGTCCCGCTTTGCGT 3’	*katE* inactivation testing	This study
Post-katE	5’ CTGCCTGATGCGCTTCGCTTA 3’	*katE* inactivation testing	This study
Pre-ahpC	5’ GCCGCTGGCGGTGCAAAGTTCAC 3’	*ahpC* inactivation testing	This study
Post-ahpC	5’ GCCGCAGCACCCGAAGAATTA 3’	*ahpC* inactivation testing	This study
Pre-sodA	5’ GTGGGTGATTTGCTTCACATCT 3’	*sodA* inactivation testing	This study
Post-sodA	5’ CACCGCCGTTGGCGATGGTTCAT 3’	*sodA* inactivation testing	This study
Pre-sodB	5’ CGGCAACAGGGTAAGTTCATCT 3’	*sodB* inactivation testing	This study
Post-sodB	5’ GCCGGATGCGGCGAGTGCCTTA 3’	*sodB* inactivation testing	This study
Pre-sdhC	5’ CTGGAAGCAGTGTTTTGCATGA 3’	*sdhC* inactivation testing	This study

Post-sdhC	5’ GCGTCAGGACGATAGCGGTA 3’	*sdhC* inactivation testing	This study
Pre-oxyR	5’ GCCGCTCCGTTTCTGTGAGCAAT 3’	*oxyR* inactivation testing	This study
Post-oxyR	5’ GGCGGCAACACTATTGAGTA 3’	*oxyR* inactivation testing	This study
Pre-rpoS	5’ CCGTAAACCCGCTGCGTTAT 3’	*rpoS* inactivation testing	This study
Post-rpoS	5’ TGGGCGGTAATTTGACCTTA 3’	*rpoS* inactivation testing	This study
K1	5´ -CAGTCATAGCCGAATAGCCT-3´	Genes inactivation testing	(3)
K2	5´ -CGGTGCCCTGAATGAACTGC-3´	Genes inactivation testing	(3)
Kt	5´-CGGCCACAGTCGATGAATCC-3´	Genes inactivation testing	(3)
Phages			
P1 phage		Genes inactivation	
Plasmids			
pCP20		Excision of the kanamycin resistance cassette	(3)
pMSrecA-gfp		recA-gfp reporter promoter fusion	(1)

Figure S1. Monitoring the SOS response in engineered *E. coli* BW25113 with *recA* gene inactivation and detoxification system inactivation for all combinations used in this study. Ciprofloxacin induces DNA damage leading to activation of the SOS response. Ciprofloxacin-induced GFP expression from the pMSrecA-gfp vector was used to display *recA* promoter activity (SOS induction) after the addition of a fluoroquinolone (ciprofloxacin). Pictures of the disc diffusion method with ciprofloxacin (5 µg) at 24 hours are shown. Green fluorescence indicates induction of SOS response.

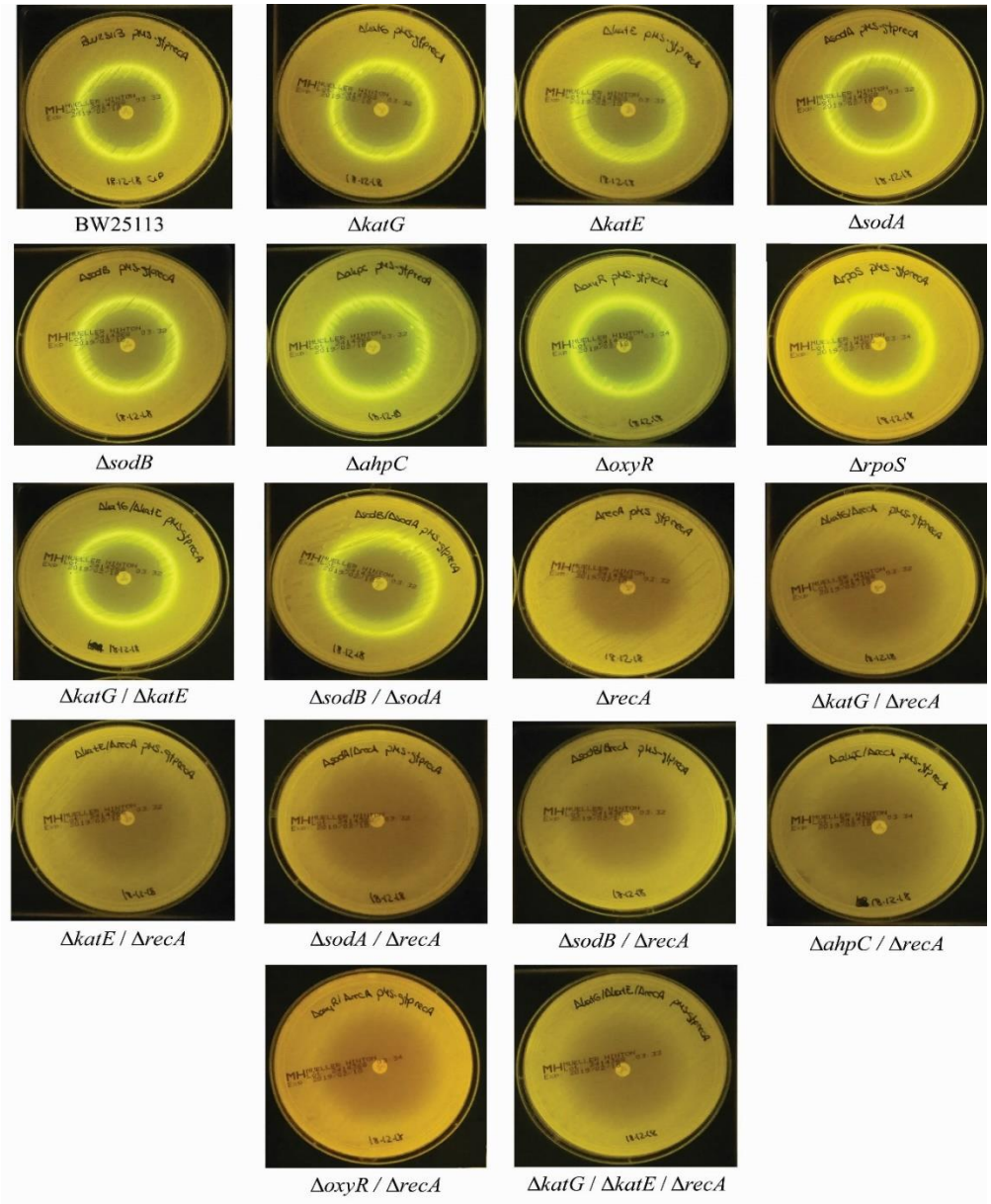


Figure S2. Growth curves for *E. coli* BW25113 and all genetic combinations for groups with ciprofloxacin concentrations of 0.0005 (A), 0.001 (B) and 0.002 mg/L (C, D, E, F and G) during 24 hours. Data are represented as the mean of at least three independent measurements. Growth curves of all constructions was similar in non-antibiotic control (data not shown).

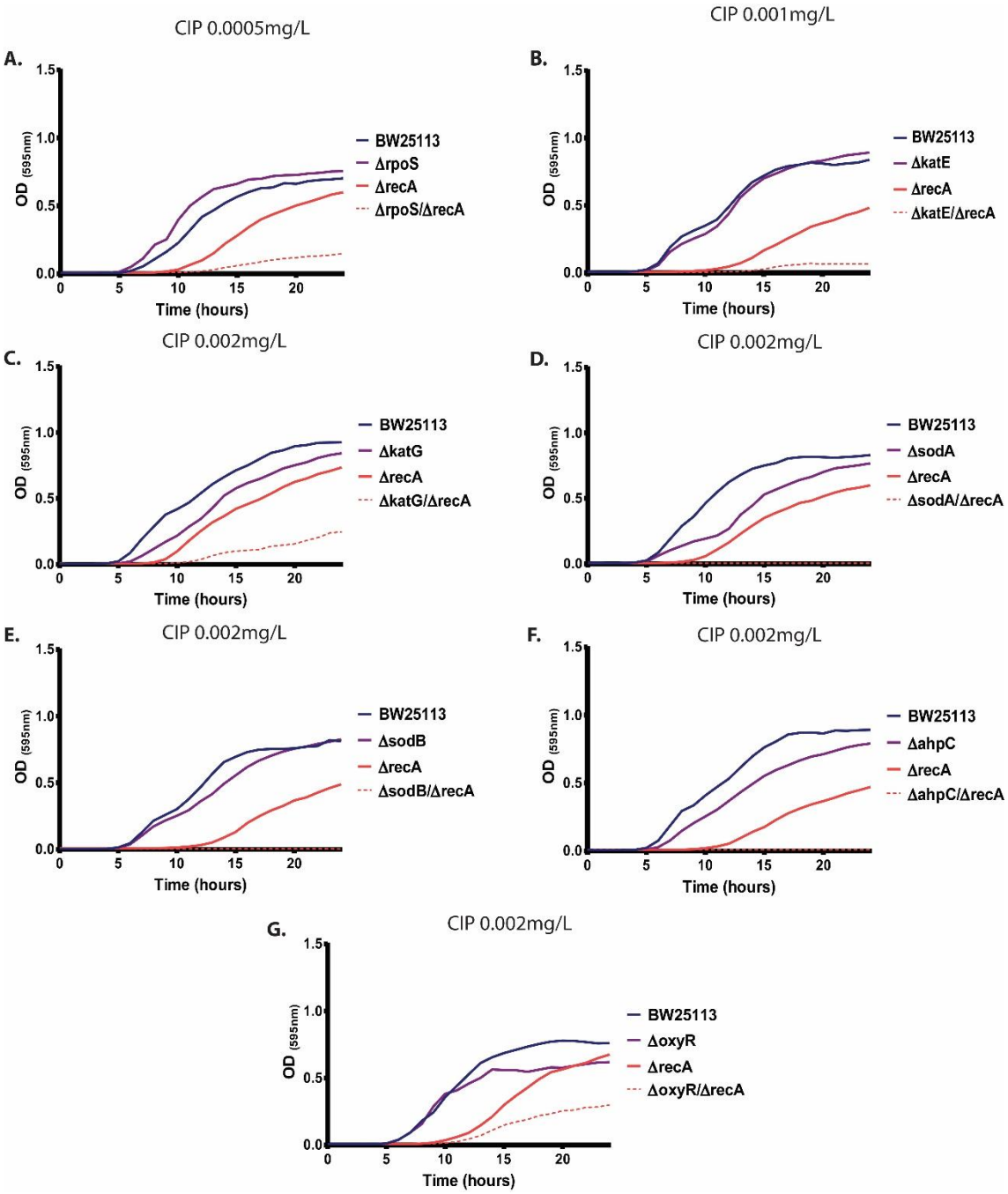


Figure S3. Viable bacterial counts of *E. coli* BW25113 with *recA* gene and detoxification system inactivation (*katG*, *sodA* and *ahpC*) combinations by groups in time-kill assays at ciprofloxacin (CIP) concentrations of 1xMIC *E. coli* BW25113 (0.015 mg/L) during 24 h. Data are represented as the means of at least three independent measurements. Time-kill curves of all constructions was similar in non-antibiotic control (data not shown). Standard deviations are shown (values not represented are inside the symbol). CIP, ciprofloxacin.

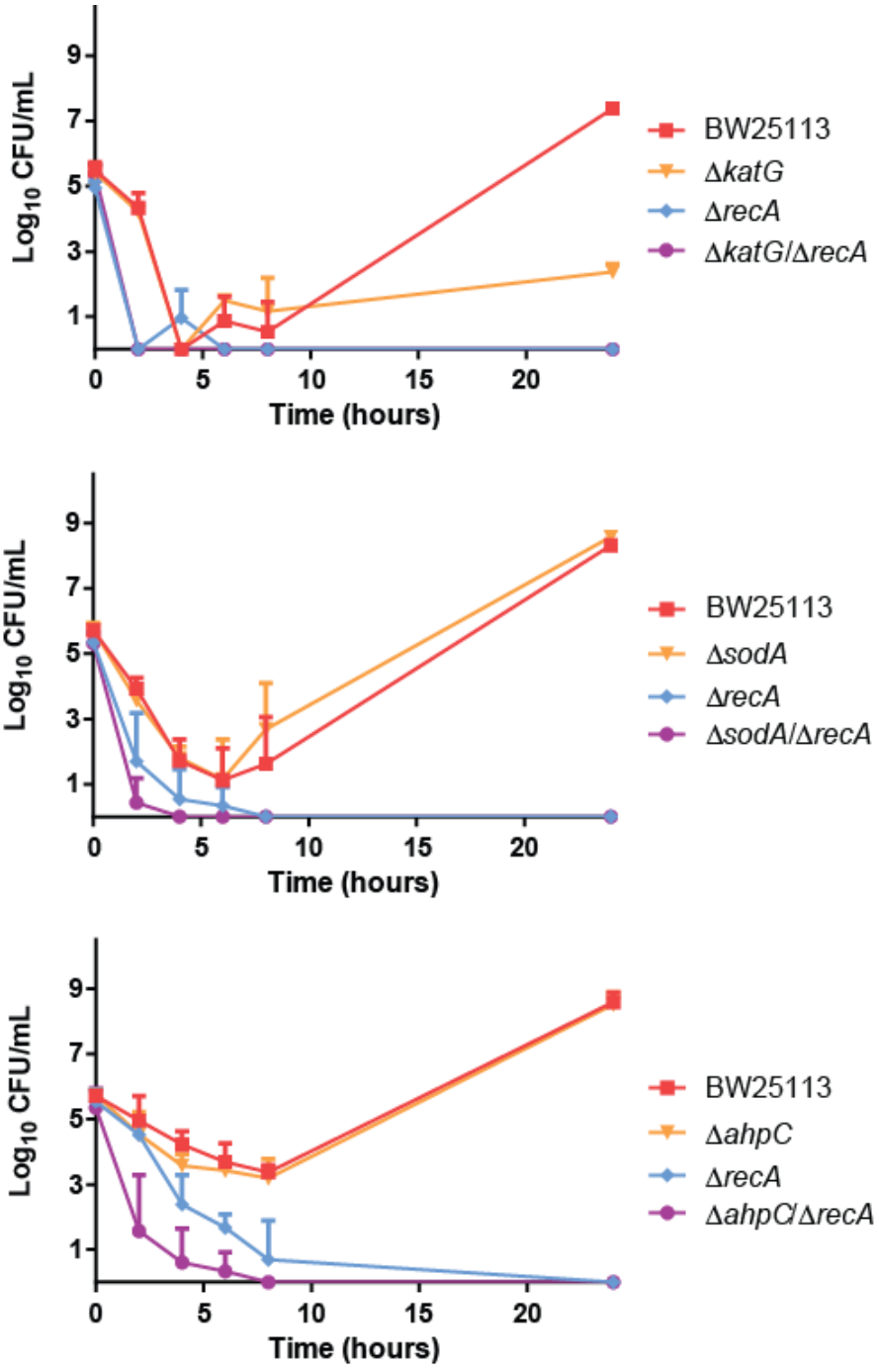


Figure S4. Bactericidal activity of quinolones is diminished with L-Ascorbic acid pH 6.8 pretreatment even if *recA* gene and detoxification systems, in combination, are suppressed. Viable bacterial counts of *E. coli* Δ*recA*, *E. coli* Δ*katG*/Δ*recA*, *E. coli* Δ*sodA*/Δ*recA* and *E. coli* Δ*ahpC*/Δ*recA* in time-kill assays at ciprofloxacin (CIP) concentrations of 1xMIC Δ*recA* (0.004 mg/L) with or without pretreatment with L-Ascorbic acid (50mM) at 4 hours. Data are represented as the means of at least three independent measurements. *E. coli* BW25113 was not included due to the antibiotic concentration used was under its MIC, so that its behaviour was similar to non-antibiotic control. Error bars represent standard deviations. Significant P values are noted (*: compared to strain without treatment with $p<0.001$; #: compared to strain with ciprofloxacin treatment with $p<0.01$).

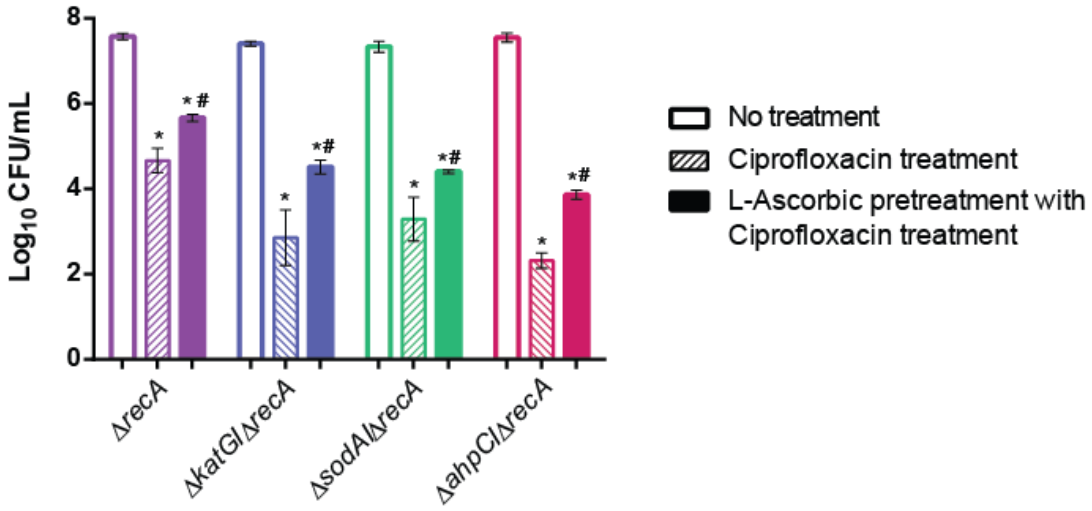


Figure S5. Combination of *recA* gene inactivation and detoxification system suppression inactivation increases ROS production in isogenic *E. coli* strains under selective quinolone concentrations. ROS measurement for *E. coli* and all genetic combinations with ciprofloxacin concentrations of 1xMIC of Δ*recA* (0.004 mg/L) at 24 h. Data were normalized to a no-dye control (background fluorescence) and OD595nm (bacterial density) and are represented as the mean of at least four independent measurements. ROS production of all constructions was similar in non-antibiotic control (data not shown). Error bars represent standard deviations. Significant P values are noted (*, <0.05; ***, <0.001).

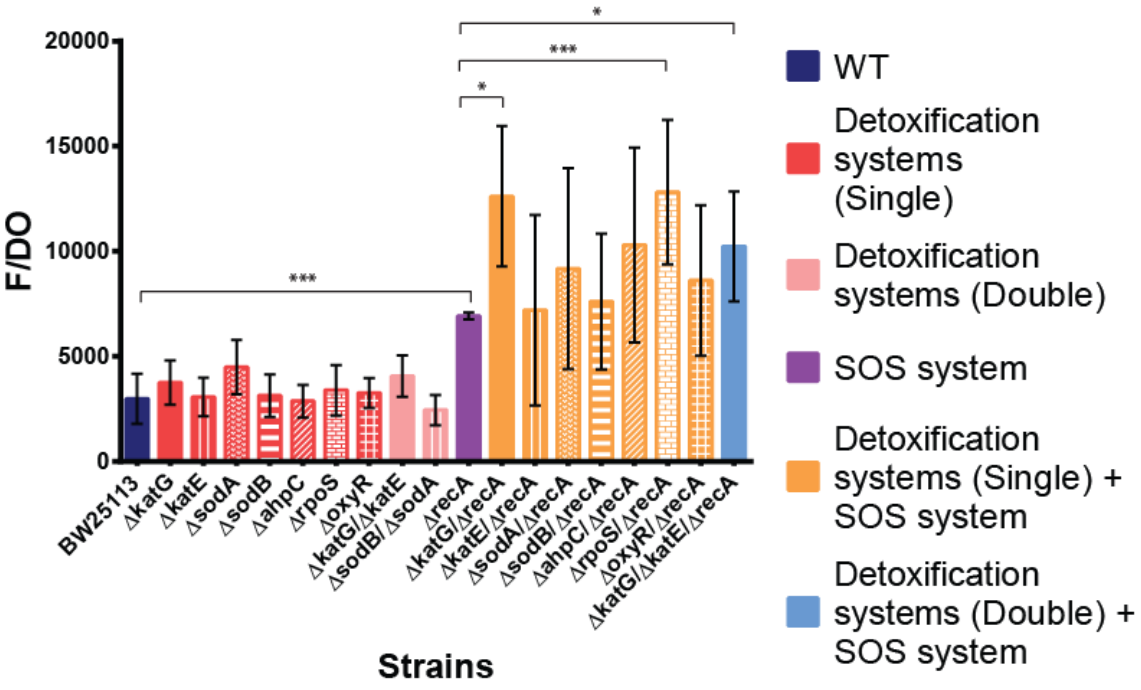
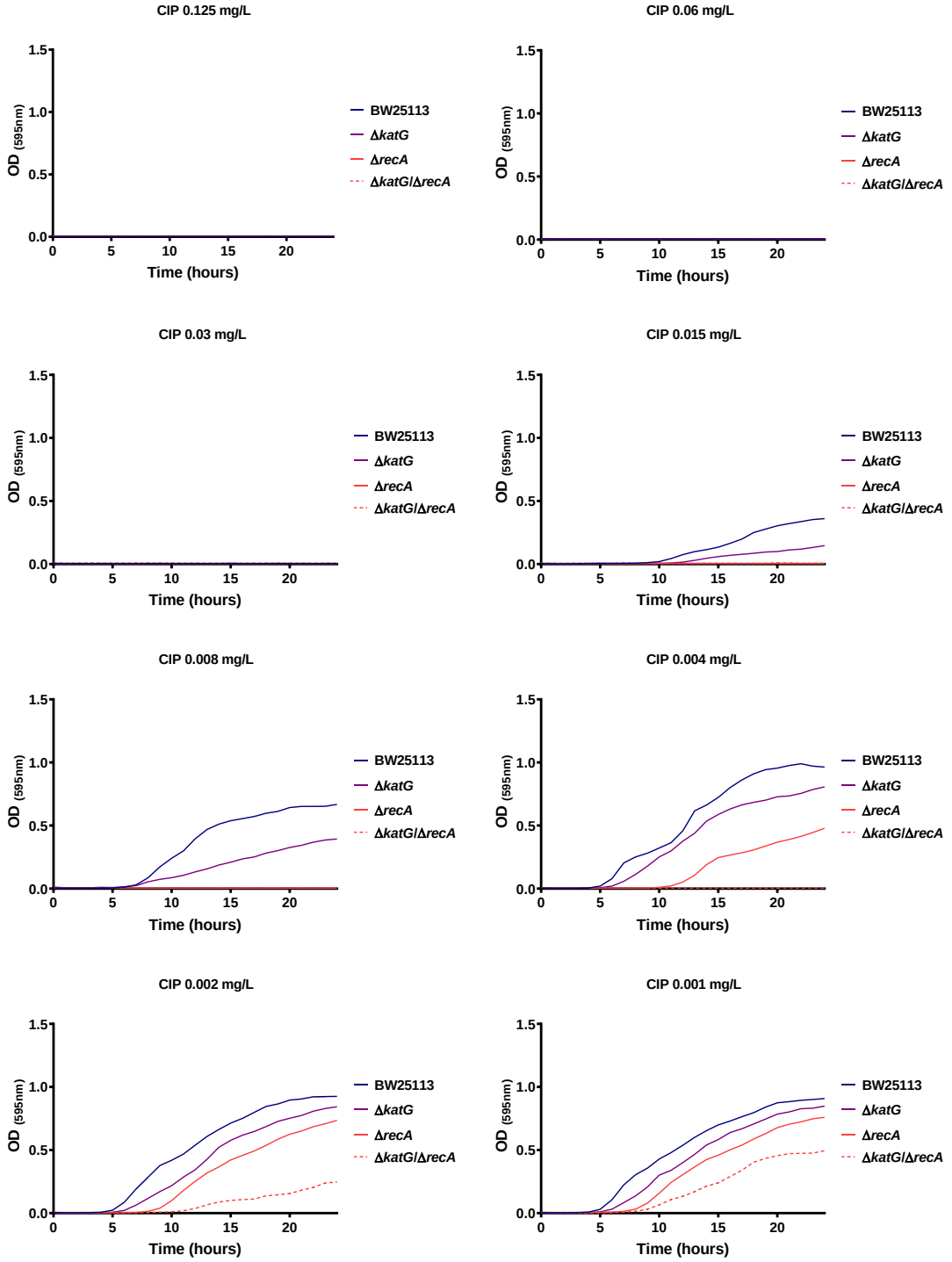


Figure S6. Growth curves for *E. coli* BW25113 with *recA* gene and *katG* detoxification system inactivation alone or in combinations with ciprofloxacin concentrations from 8xMIC to 1/256xMIC of *E. coli* BW25113 during 24 hours. Data are represented as the mean of at least three independent measurements. Growth curves of all constructions were also performed without antibiotic in parallel (Untreated). Not growth was observed at 0.125-0.03 mg/L ciprofloxacin concentrations.



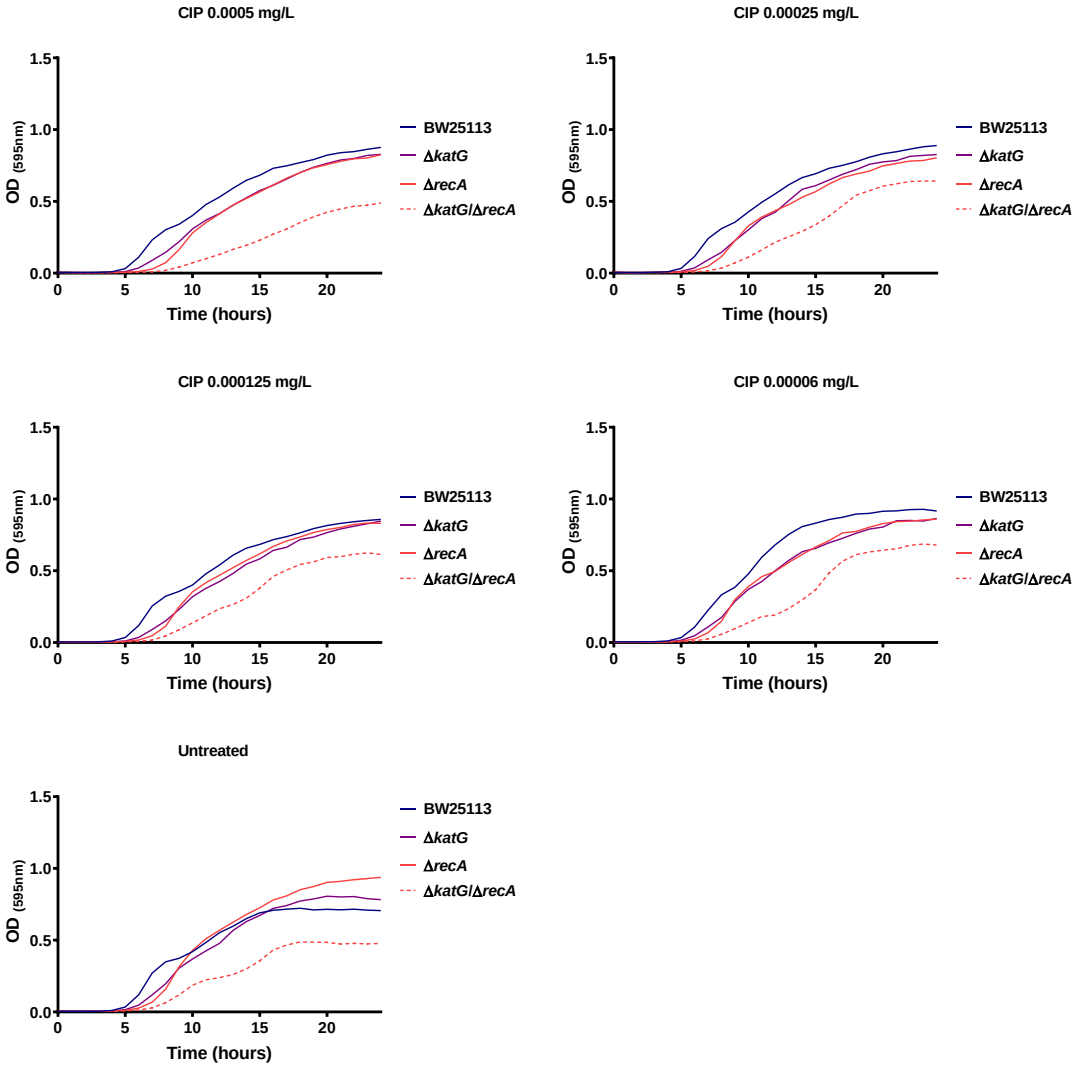
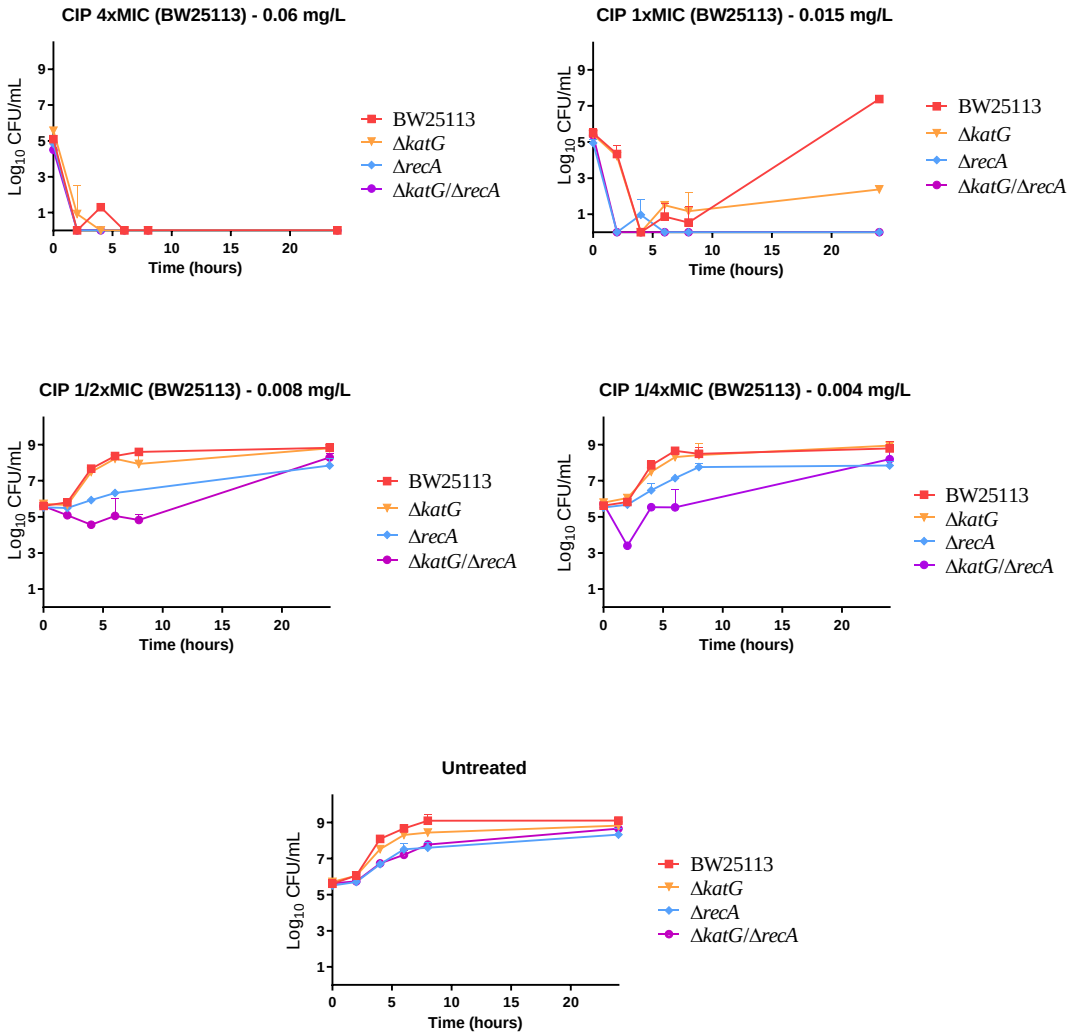

CIP 0.0005 mg/L
CIP 0.00025 mg/L
CIP 0.000125 mg/L
CIP 0.00006 mg/L
Untreated
OD (595nm)
Time (hours)
BW25113
ΔkatG
ΔrecA
ΔkatG/ΔrecA

Figure S7. Viable bacterial counts of *E. coli* BW25113 with *recA* gene and *katG* detoxification system inactivation alone or in combinations in time-kill assays at ciprofloxacin (CIP) concentrations of 4xMIC (0.06 mg/L), 1xMIC (0.015 mg/L), 1/2xMIC (0.008 mg/L) and 1/4xMIC (0.004 mg/L) of *E. coli* BW25113 during 24 h. Data are represented as the means of at least three independent measurements. Time-kill curves of all constructions were also performed without antibiotic in parallel (Untreated). Standard deviations are shown. CIP, ciprofloxacin.



REFERENCES

1. Zaslaver A, Bren A, Ronen M, Itzkovitz S, Kikoin I, Shavit S, Liebermeister W, Surette MG, Alon U. 2006. A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. Nat Methods 3:623-638.

2. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H. 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol Syst Biol 2:2006.0008.

3. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A 97:6640-6645.