1 **Supplemental File** (contains Text, References and Tables S1, S2, S3)

2 **Experimental procedure**

3 **Bacterial strains, culture and selection conditions, and antibiotics**

4 Selection and screening was carried out in E. coli K-12 substrate MG1655 (NCBI uid57779) and in the
5 ciprofloxacin resistant mutants derived from it. All manipulations with bacterial cultures were done on
6 Luria–Bertani (LB) agar plates or in LB broth. A mutant resistant to high concentration of ciprofloxacin (MIC
7 ≥ 24 ug/ml) was selected in 4 steps by successive passaging of overnight cultures from a preceding selection
8 stage on plates supplemented with ciprofloxacin at 0.03 μg/ml (step 1), 0.25 μg/ml (step 2), 1 μg/ml (step
9 3) and 8 μg/ml (step 4). Clones selected from each step, designated rCip-0.03, rCip-0.25, rCip-1 and rCip-8,
10 were frozen from overnight cultures and kept in 15% Glycerol at -80 °C.

11 MFD*pir* strain (1) (a gift from Russel Monds, Stanford University) used in conjugative transposon transfer
12 (see below) was grown in the medium supplemented with 0.3 mM diaminopimelic acid, DAP (Sigma-
13 Aldrich, St. Louis, MO).

14 Kanamycin (Teknova, Hollister, CA) was added when necessary to liquid or solid media to a final
15 concentration of 50 μg/ml. Ciprofloxacin (LKT laboratories, St. Paul, MN) was used in a range of final
16 concentrations between 10 – 30,000 ng/ml.

17

18 **Determining the minimal inhibitory concentration (MIC) of ciprofloxacin**

19 MIC values of ciprofloxacin for parental and resistant strains were determined using a procedure described
20 by Wiegand et al. (2). Briefly, cultures were maintained in early- to mid-exponential phase for at least 6
21 generations before the MIC test. Serial dilutions ranging from 10^2 – 10^7 cells per ml were prepared in saline
22 and 10 μl suspension of each dilution was spotted on LB agar plate containing the antibiotic at a given
23 concentration. MIC was estimated as the lowest concentration of ciprofloxacin in a series of concentrations
24 (with 2-fold increment, for concentrations ≤ 500 ng/ml, and with 1 μg/ml increment, for concentrations ≥ 1
25 μg/ml) at which patches with 10^4 and fewer cells showed no visible growth after 48 h incubation at 37 °C.
26 These estimates have been corroborated by a liquid assay. The effects of knock-outs and deletions on MIC
27 were tested in MG1655 and BW25113 genetic backgrounds.

28

29 **Conjugation and construction of a transposon library**

30 The plasmid pEB001 (3) carrying a modified version of the transposon delivery vector pMiniHimar RB1 (4)
31 was transformed into MFD*pir*. The resulting strain MFD*pir*-pEB001 was used as a donor in the following
32 conjugation procedure. An overnight culture of the rCip-8 mutant strain (1.4 ml) was heat shocked at 42 °C
33 for 5 min and mixed with a pellet obtained by centrifuging 140 µl of an overnight culture of MFD*pir*-
34 pEB001. The mixture was centrifuged and resuspended in 100 µl of LB+DAP, then 10 µl was spotted on an
35 LB+DAP agar plate. After mating at 30 °C overnight, the cells were scraped from plates, resuspended in LB,
36 and spread on LB with 50 µg/ml kanamycin for selection and recovery. Approximately 60,000 colonies were
37 collected for a transposon library. The library was stored at -80 °C in LB supplemented with kanamycin (50
38 ug/ml) and 15% glycerol. A transposon library in the MG1655 parental (ciprofloxacin susceptible) strain was
39 constructed in the same way.

40

41 **Library screening**

42 An aliquot of frozen transposon library stock of the strain rCip-8 was thawed at 37 °C and diluted to OD_{600}
43 of 0.16 using LB with 50 µg/ml kanamycin. The culture was incubated for 1 h at 37 °C with aeration at 260
44 rpm, at which point a $\mathbf{t_0}$ sample was collected. The rest of the culture was diluted to OD_{600} of 0.05 and
45 divided into two sub-cultures. Ciprofloxacin was added to a final concentration of 4 µg/ml to one and no
46 drug was added to the other. Sub-cultures were maintained in exponential phase, with or without
47 ciprofloxacin, by repeated dilutions in a constant volume. A $\mathbf{t_1}$ samples for both the drug exposed and no-
48 drug sub-cultures were collected after 10 doublings. The library of MG1655 was processed in a similar way,
49 except that the culture was propagated without ciprofloxacin in the medium and, as a result, only one $\mathbf{t_1}$
50 sample had to be collected. Untreated controls were processed in the same way. Pelleted samples were
51 stored at -80 °C until the next step. The screen was done in 3 replicates, where t_0 samples were obtained
52 from different aliquots of the original libraries.

53

54 **Sample preparation for Illumina sequencing and data processing**

55 Genomic DNA was extracted from the t_0 and t_1 samples using the Wizard® genomic DNA purification kit
56 (Promega, Madison, WI) and processed as previously described (5) except that the adaptors had the
57 following sequences:

58 p5_side_generic: /5Phos/CTGTCTCTTATACACATCTGACGCTGCCGACGA

59 p5_side_NN:TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGNN.

60 The enrichment PCR was carried out using primers with following sequences:

61 p5_primer 1: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGAGACCGGGGACTTATCATCCAACCTGT

62 p7_side_Magellan_enrichment_primer: 5'-TCGTCGGCAGCGTCAGATGTGTATAA.

63 18 samples were indexed using forward: 5'-AATGATACGGCGACCACCGAGATCTACAC[i5]TCGTCGGCAGCGTC

64 and reverse indexing primer: 5'-CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG.

65 Pooled 18 libraries were sequenced in one lane as 50 bp single end reads using HiSeq2500 at the University

66 of Minnesota Genomics Center.

67 Following de-multiplexing, the reads were trimmed and the transposon-specific sequence was removed

68 using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html). Resulting reads were mapped to

69 the MG1655 genome using Bowtie 2 (6, 7). The SAM files of mapped reads were converted to BAM format

70 and sorted using SAMTOOLS (8). The sorted BAM files were converted to BED files and intersections with

71 annotated ORF's were enumerated using BEDTOOLS (9). A final dataset for each sample contained two

72 metrics for each ORF: counts - the number of times transposon-linked reads overlapped with the ORF; and

73 coverage – the fraction of base pairs within the ORF that overlapped with the reads.

74

75 **Experimental design**

76 The objective was to identify genes whose inactivation in the genome of a highly ciprofloxacin resistant *E.*

77 *coli* would lead to sensitization of the resistant strain to the drug. By definition, antibiotic resistant mutants

78 can grow and divide when the antibiotic is present in the media at below MIC levels. Mutations that lead to

79 the reduction of MIC may interfere with growth and/or division at antibiotic concentrations that are fully

80 tolerated by the resistant parent strain. Therefore, cells carrying the MIC lowering mutations will be

81 relatively disadvantaged in presence of the antibiotic, compared to the cells that do not carry such

82 mutations, and will be depleted from the antibiotic exposed population over time. However growth

83 deficiencies of individual mutant cells may or may not be related to the effect of an antibiotic. Hence, it is

84 imperative to account for such antibiotic unrelated growth variations in an experimental design (Fig. 1).

85 Thus, we set out to determine the abundance of mutant alleles in the samples of the drug resistant mutant

86 before exposure to ciprofloxacin, after exposure to the drug for 10 generations and after 10 generations

87 without the drug. Additionally, we have examined the mutant allele abundances in a ciprofloxacin sensitive,

88 wild type strain from which the resistant strain has originated. To achieve the experimental objective, we

89 had to identify mutant alleles whose abundance in a population of ciprofloxacin-exposed cells decreased
90 after 10 generations significantly more than in populations grown without the antibiotic.

91 Quantifiable mutant alleles were generated by saturation transposon mutagenesis using a modified
92 mariner transposon (3, 4). Following transposon mobilization, approximately 60,000 transposon insertion
93 mutants of the ciprofloxacin resistant strain and about the same number of its drug sensitive parent have
94 been pooled into two separate mutant libraries. 10% (by biomass) of the libraries were used as t_0 samples
95 in the selection experiments, to improve the chances of representative sampling of the mutant
96 populations. 1.8 - 4.4 million reads were mapped to 4516 ORF's annotated in the genome of MG1655
97 (Table 1S), with an average number of insertions per gene varying between 400 and 958. To assess the
98 genetic quality of the libraries, we evaluated the distribution of insertions in two groups of genes: a set of
99 essential genes in LB (264 genes), consistent between two independent studies of essentiality of *E. coli*
100 genes in rich growth media (10, 11), and its complement (4252 genes), defined as all genes in the genome
101 that did not make it onto the common set of essential genes. The qualitative analysis revealed that the
102 transposon insertions were found on average much less frequently (at least an order of magnitude) and in a
103 smaller ORF portion (about 10 times) in essential genes compared to non-essential ones (Table 1S).
104 Furthermore, we observed, using average metrics as classification cut-offs, that less than 1.1% of essential
105 genes were likely to be classified as non-essential and at most 5.1% of non-essential genes were likely to be
106 classified as essential. This statistical summary indicates that the distribution of mutations in sampled
107 libraries is sufficiently random, representative, and comports with biological expectations.

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142 **Table S1. Benchmarks of transposon insertion libraries before selection**

	Metric	Gene class	$Cipt_{01}$+D[5]	$Cipt_{02}$+D	$Cipt_{03}$+D	$Cipt_{01}$	$Cipt_{02}$	$Cipt_{03}$	$Wt\text{-}t_{01}$	$Wt\text{-}t_{02}$	$Wt\text{-}t_{03}$
Mean	count[1]	Nes[2]	234.1	234.2	234.08	234.11	234.16	234.15	234.13	234.05	234.15
		Es[3]	16.92	15.43	17.42	17.04	16.19	16.31	16.7	18.14	16.35
	breadth	Nes	21.29%	19.18%	21.04%	21.42%	21.65%	24.02%	30.72%	27.46%	28.62%
		Es	2.81%	2.14%	2.55%	2.49%	2.74%	3.42%	3.22%	2.99%	3.16%
Median	count	Nes	153.69	151.25	158.36	153.33	154.48	154.58	132.94	128.44	132.72
		Es	0.63	0.62	0.48	0.47	0.39	0.69	1.68	1.66	2.06
	breadth	Nes	20.27%	18.32%	20.07%	20.53%	20.61%	23.12%	30.47%	26.93%	28.32%
		Es	1.85%	0.61%	1.42%	1.42%	1.41%	2.20%	1.68%	1.39%	1.64%
fr_{nes} <Mean(Es_count&br)[4]			0.044	0.051	0.048	0.046	0.048	0.038	0.033	0.038	0.037
fr_{es} >Mean(Nes_count&br)			0.004	0.008	0.008	0.008	0.008	0.008	0.008	0.004	0.011
Total reads mapped to ORFs			3.19E+06	1.62E+06	2.06E+06	2.14E+06	2.59E+06	4.33E+06	2.38E+06	1.81E+06	1.94E+06

143 1 – normalized to one million reads; 2 – Non-essential genes (Nes); 3 – essential genes (Es); 4 – fraction of all non-
144 essential genes in the set with insertion count and breadth of coverage less than the corresponding means for the
145 essential gene set; 5 – a time point 0 sample of rCip-8.0 mutant with Ciprofloxacin (+D) added.

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151 **Table S2. Summary of predicted suppressors and their verification**

Class[1]	Number of mutants	Number with reduced MIC[2]	True Positives, %
1	26	16	61.54%
2	26	3	11.54%
Total	52	19	36.54%

152 [1] - see text for class definitions; [2] - at least 2-fold reduction was observed in multiple tests.

153

154 **Table S3. Suppression of ciprofloxacin resistance in single-gene deletion mutants**

Name of the deleted gene	t0 Counts, average	t0 Breadth of coverage, average	Fitness Score	MIC of deletion mutant , average	Predicted suppressor in rCip-8	Suppressor in sensitive wild type	Confirmed inference from the present study
xseB	0.05	0.01	0	12	No	Yes	FALSE NEGATIVE
ruvA	0.24	0.01	0	5	No	Yes	FALSE NEGATIVE
fis	0.27	0.03	0	16	No	Yes	FALSE NEGATIVE
ruvC	0.44	0.05	0	6	No	Yes	FALSE NEGATIVE
rseA	241.32	0.22	0.6	16	No	Yes	FALSE NEGATIVE
ygcO	142.38	0.23	0.9	32	No	Yes	TRUE NEGATIVE
ihfA	145.95	0.22	1.1	32	No	Yes	TRUE NEGATIVE
ycjU	170.03	0.13	1.1	30	No	Yes	TRUE NEGATIVE
rimK	191.21	0.14	1.2	30	No	Yes	TRUE NEGATIVE
yciT	126.2	0.2	1	28	No	Yes	TRUE NEGATIVE
ybgF	141.57	0.21	1.4	28	No	Yes	TRUE NEGATIVE
ybgC	175.96	0.37	0.9	32	No	Yes	TRUE NEGATIVE
qmcA	310.54	0.3	0.9	28	No	Yes	TRUE NEGATIVE
yjjY	319.88	0.49	0.7	36	No	Yes	TRUE NEGATIVE
hrpA	387.16	0.14	0.9	28	No	Yes	TRUE NEGATIVE
yciM	203.34	0.23	2.4	16	Yes	Yes	TRUE POSITIVE
dksA	109.22	0.15	3.1	16	Yes	Yes	TRUE POSITIVE
hfq	121.37	0.16	5.1	8	Yes	Yes	TRUE POSITIVE
recG	144.51	0.11	6.4	6	Yes	Yes	TRUE POSITIVE
recB	146.7	0.06	8.2	4	Yes	Yes	TRUE POSITIVE
recA	151.05	0.11	3.2	8	Yes	Yes	TRUE POSITIVE
recC	163.14	0.09	9.2	4	Yes	Yes	TRUE POSITIVE
xseA	176.05	0.19	3.6	12	Yes	Yes	TRUE POSITIVE
tolC	236.53	0.16	27.9	1	Yes	Yes	TRUE POSITIVE
recN	506.36	0.23	2.4	16	Yes	Yes	TRUE POSITIVE
acrA	513.53	0.22	23.6	1	Yes	Yes	TRUE POSITIVE
acrB	735.64	0.14	33.6	1	Yes	Yes	TRUE POSITIVE
uvrD	796.94	0.22	4.1	8	Yes	Yes	TRUE POSITIVE
yciS	202.23	0.21	4.1	10	Yes	No	TRUE POSITIVE
miaA	207.57	0.22	3.3	16	Yes	No	TRUE POSITIVE
hofO	212.15	0.22	1.9	20	Yes	No	TRUE POSITIVE
ygaH	272.31	0.25	2.3	16	Yes	No	TRUE POSITIVE
lpxM	292.39	0.24	2.8	16	Yes	No	TRUE POSITIVE
marA	341.54	0.46	6.3	8	Yes	No	TRUE POSITIVE

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