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Supplemental Information

A Strategic Target Rescues Trimethoprim Sensitivity in *Escherichia coli*

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Supplementary Information

Supplementary Figures

Figure S1: Growth of WT and laboratory-evolved TMP-resistant 32xR *E. coli* [related to Figure 1]: Growth curves of 32xR1 and 32xR2 *E. coli* in presence (-T) and absence of 16 µg/mL TMP; and respective *E. coli* K12 MG1655 parents (WT1 and WT2) are shown. A_{600} recorded at each hour is shown as mean ± SD. The 32xR strains grow only marginally slower as compared to their respective WT parents and there is no significant difference in growth in presence and absence of 16 µg/mL TMP.

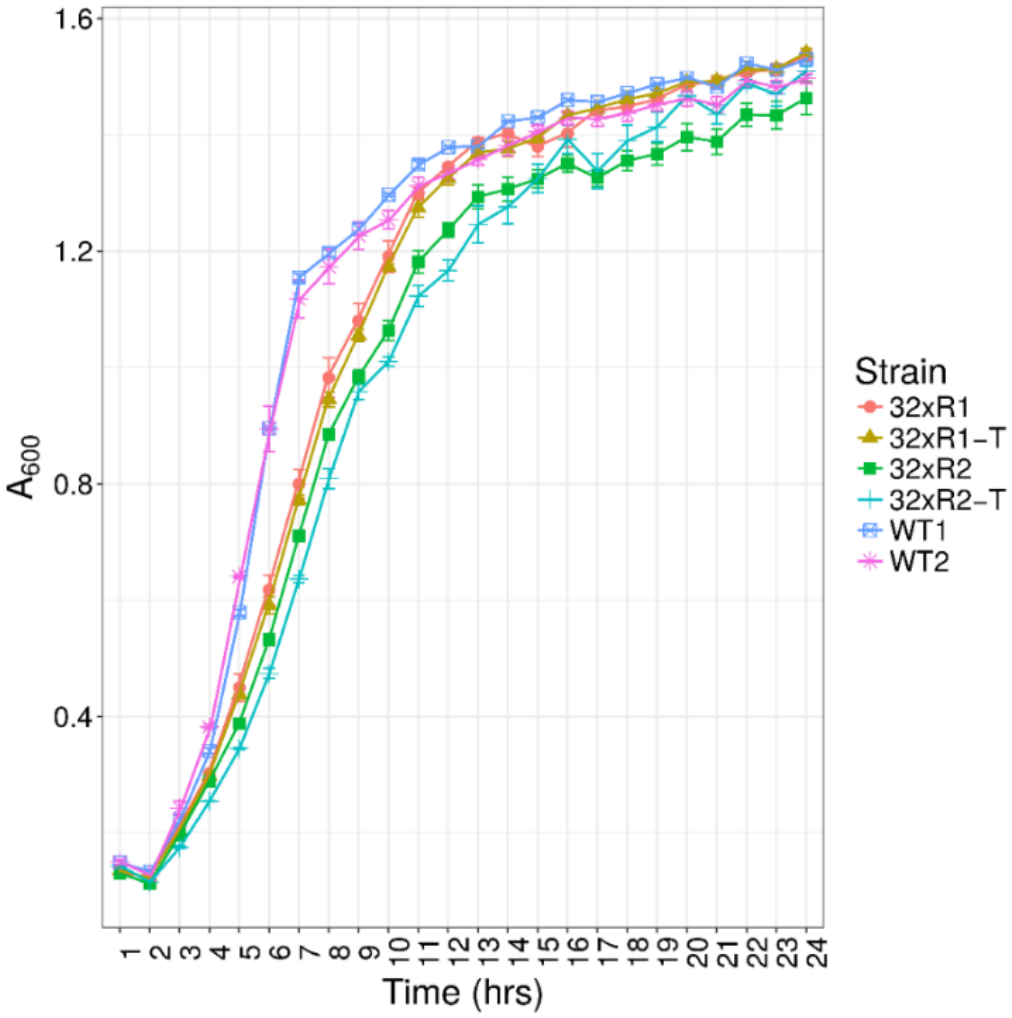


Figure S2: Clusters identified in 32xTopNet [related to Table 1]: ClusterONE (Clustering with Overlapping Neighbourhood Expansion (Nepusz et al., 2012)) was used to identify clusters based on edge-weights. ClusterONE identifies clusters with overlapping nodes. For example, if a gene pair A-B has a higher edge-weight and so does the pair A-C but not the pair B-C, then gene A will be observed in two clusters, one which has gene B and its interactions and another which has gene C and its interactions. Therefore, multiple clusters containing the same genes are observed. 26 clusters were identified.

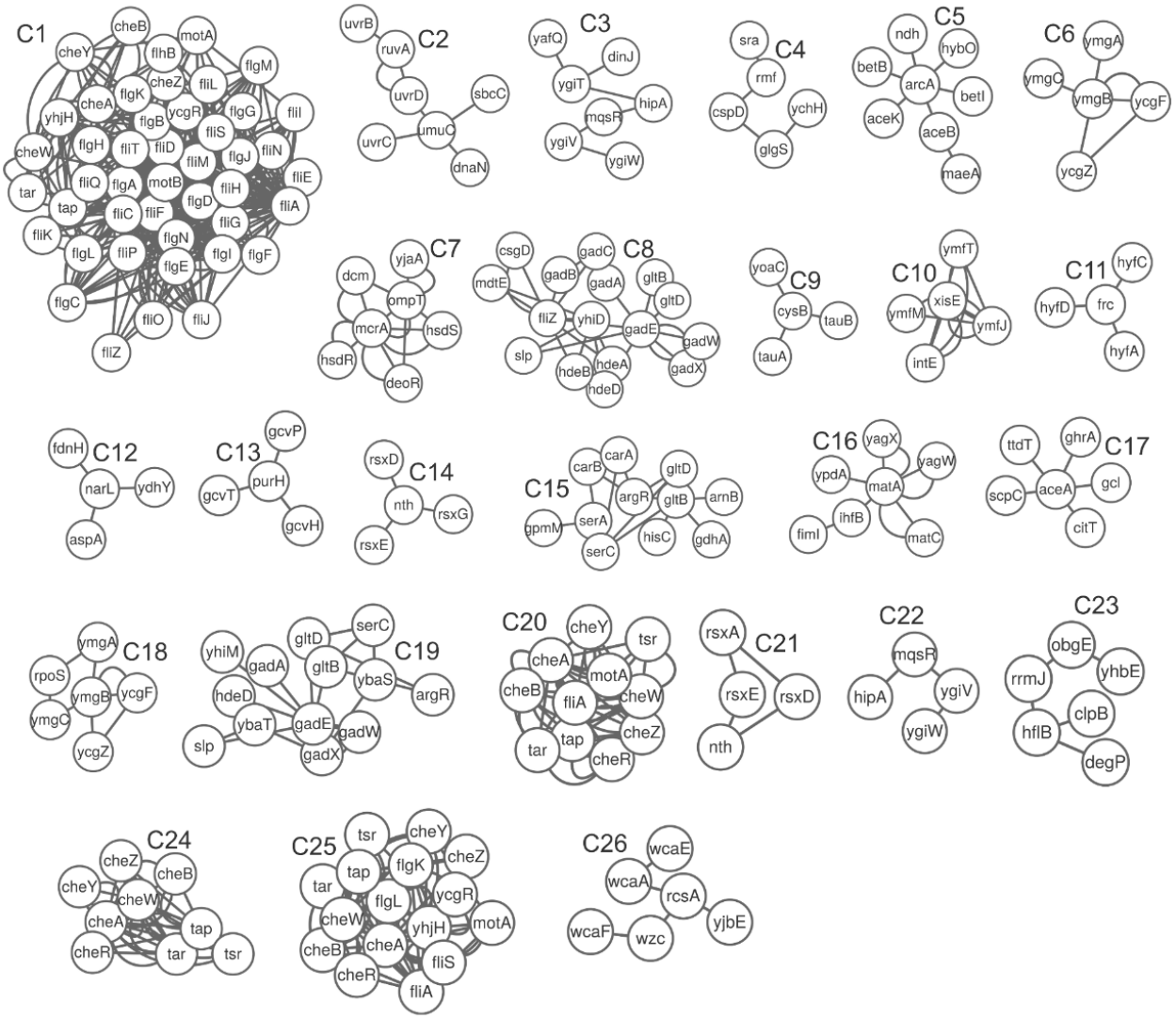


Figure S3: Biofilm formation [related to Table 1, Figure 1 and Figure 2]: (a) Biofilm quantification by crystal violet staining (A_{590} data plotted as mean ± SD) showed that biofilm production by the 32xR strains both in the absence and presence of 16 µg/mL TMP was higher as compared to WT. (b) Scanning electron microscopy (SEM) images at 4000X of *E. coli* biofilms showed that 32xR *E. coli* clump together in a biofilm matrix whereas WT appear mostly as separate cells. The clumping in each field is demarcated for ease of viewing.

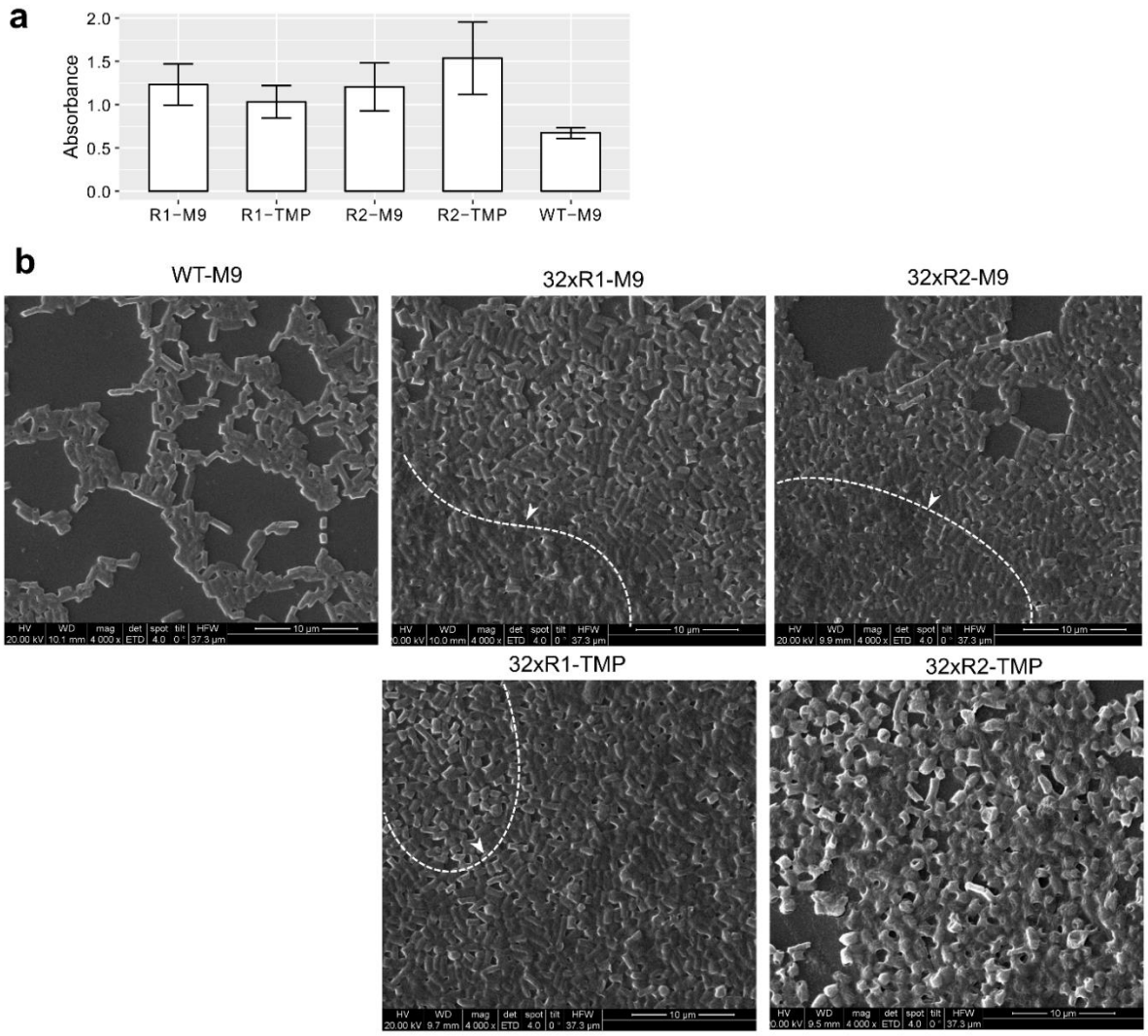


Figure S4: Confirmation of *glyA* knockouts [related to Figure 2C]

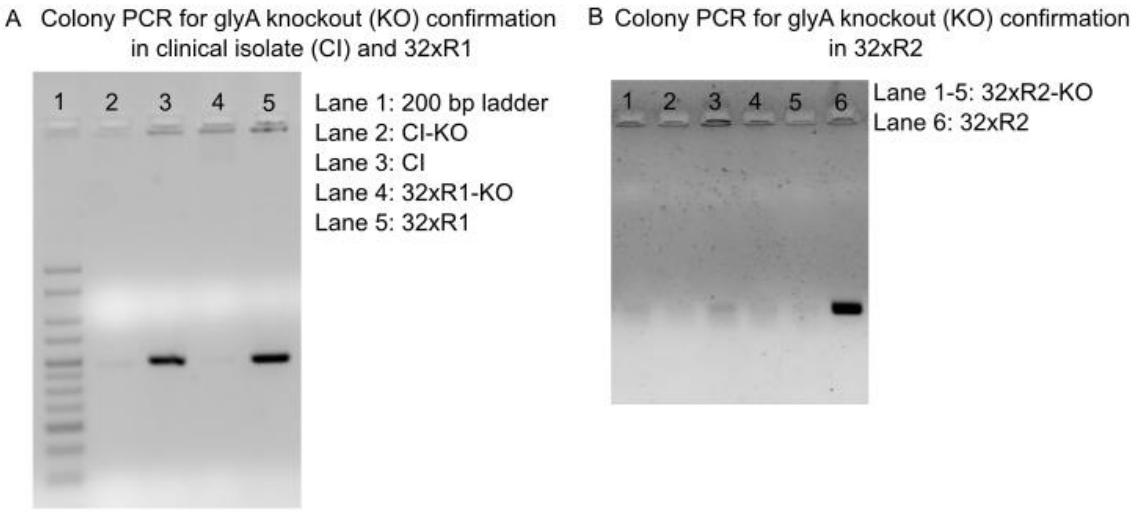


Figure S5: Growth curves of 32xR1, 32xR2 and CI and their respective *glyA* knockouts [related to Figure 2]: Growth in the absence of TMP was profiled for 32xR strains and clinical isolate-CI (red) and their respective Δ*glyA* (black) over 24 hours. $\log_{10}$(CFU/mL) is the average of two biological replicates.

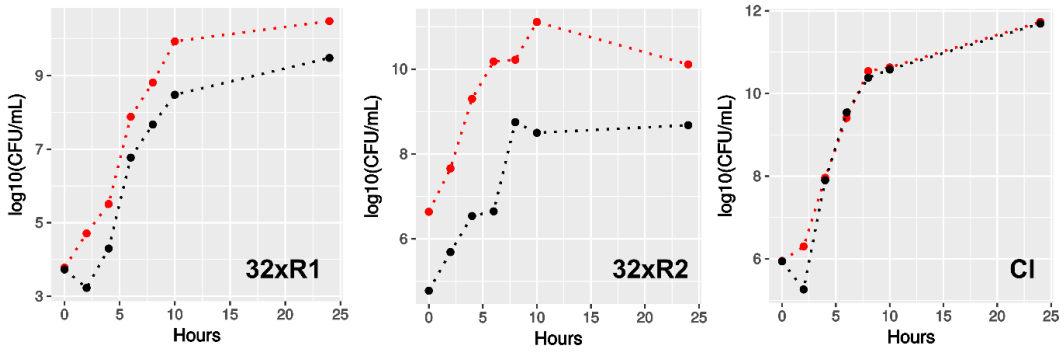
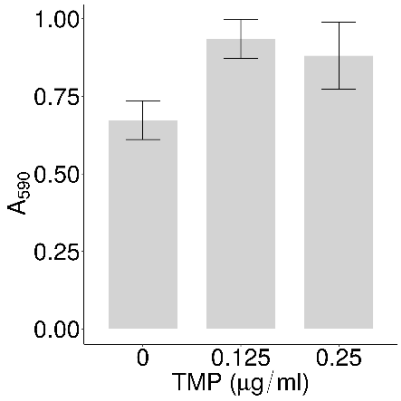


Figure S6: Biofilm formation by WT in response to TMP stress [related to Figures 1 and Figure 3]: Biofilm quantification by crystal violet staining (A_{590} data plotted as mean ± SD) showed that biofilm production in WT increases upon exposure to sub-inhibitory, but stress inducing, TMP concentrations i.e. 0.25xMIC (0.125 µg/mL) and 0.5xMIC (0.25 µg/mL) (p-value < 0.01).



Supplementary Tables

Table S1: DEGs in the 32xR *E. coli* [related to Figure 1]: log_2FC is the mean log_2FC for the 32xR1 and 32xR2 strains

Gene	log_2FC	Gene	log_2FC	Gene	log_2FC	Gene	log_2FC	Gene	log_2FC
ais	1.81	*recN*	1.76	*yeeD*	2.55	*codB*	-1.99	*maeA*	-1.1
allB	1.61	*recX*	3.49	*yeeE*	2.88	*cyaA*	-1.21	*metE*	-2.01
aphA	1.41	*rfaB*	1.29	*yegJ*	1.33	*cybB*	-1.75	*mipA*	-1.06
aspA	2.82	*rfaI*	1.02	*yfaE*	1.18	*entD*	-1.24	*mntH*	-1.17
betB	1.87	*rfaS*	1.21	*yfbP*	1.57	*fdnH*	-1.19	*modA*	-1.73
betI	2.4	*rhsA*	1.32	*yfcV*	1.53	*fecR*	-1.18	*modB*	-1.63
cadA	1.02	*rhsD*	2.61	*yfdY*	1.21	*fepA*	-1.54	*modC*	-1.87
csiE	1.71	*ribB*	1.06	*ygcK*	1.21	*fhuE*	-2.04	*modF*	-1.2
dinG	1.26	*rmf*	1.78	*ygcL*	2.06	*fimA*	-5.72	*motA*	-4.91
dinI	2.13	*ruvA*	1.56	*ygcO*	1.03	*fimC*	-3.58	*motB*	-5.29
dinJ	1.33	*sbmC*	1.54	*ygdQ*	1.02	*fimD*	-2.42	*ndh*	-1.44
dinQ	2.83	*sfmD*	1.3	*ygiS*	2.9	*fimF*	-2.66	*ompF*	-1.35
emrE	1.35	*smpA*	1.06	*ygiT*	3.47	*fimG*	-2.62	*ompT*	-3.47
fimB	1.31	*sucA*	1.34	*ygiV*	1.24	*fimH*	-2.02	*oppA*	-1.17
fimE	1.21	*sulA*	3.57	*ygiW*	2.3	*fimI*	-4.58	*pntB*	-1.45
folA	4.27	*tauA*	1.16	*ygiZ*	1.02	*fiu*	-2.84	*pqqL*	-3.6
frc	1.1	*tauB*	1.01	*ygjN*	2.46	*flgA*	-4.89	*pyrB*	-3.06
ftnB	1.6	*tdcB*	1.33	*yhdN*	1.43	*flgB*	-5.87	*pyrD*	-1.53
gadA	2.82	*tfaE*	1.9	*yhhH*	1.42	*flgC*	-5.85	*pyrI*	-3
gadB	2.79	*tisA*	4.8	*yhiD*	3.02	*flgD*	-5.94	*rnb*	-1.11
gadC	2.15	*tisB*	5.07	*yhiM*	2.34	*flgE*	-5.55	*rsxD*	-1.1
gadE	3.55	*torA*	3.53	*yhiP*	2.35	*flgF*	-5.63	*rsxE*	-1.11
gadX	3.37	*torC*	5.91	*yhjX*	4.66	*flgG*	-5.31	*rsxG*	-1.06
galE	1.89	*torD*	3.09	*yibA*	1.17	*flgH*	-4.87	*sapA*	-1.33
glgS	1.33	*torY*	2.07	*yibD*	1.88	*flgI*	-4.69	*serA*	-3.03
gltS	1.42	*tyrP*	1.01	*yibT*	1.42	*flgJ*	-4.68	*shiA*	-1.18
glyA	1.26	*umuC*	3.95	*yibV*	2.22	*flgK*	-5.19	*speD*	-1.36
guaA	1.28	*umuD*	3.15	*yjbJ*	1.33	*flgL*	-4.7	*speE*	-1.26
hdeA	3.36	*wcaD*	1.58	*yjbM*	1.04	*flgM*	-4.73	*sufD*	-1.21
hdeB	3.14	*wcaE*	1.79	*yjbR*	1.46	*flgN*	-4.71	*tap*	-5.49
hdeD	2.74	*wcaF*	1.38	*yjeN*	1.48	*flhA*	-3.2	*tar*	-6.11
hflB	1.17	*xapR*	1.41	*yjfJ*	1.14	*flhB*	-3.99	*thrA*	-2.41
hha	1.14	*xisE*	5.66	*yjfK*	2.32	*flhE*	-3.66	*thrB*	-2.07
hlyE	1.28	*yacL*	1.51	*yjhI*	2.67	*fliA*	-5.4	*thrC*	-2.18
htrL	1.46	*yadC*	1.19	*ymfD*	1.9	*fliC*	-5.69	*trg*	-2.32
hybO	1.86	*yadI*	1.17	*ymfJ*	5.96	*fliD*	-5.47	*trpE*	-6.27
idnD	1.05	*yadK*	1.81	*ymfL*	4.8	*fliE*	-4.31	*tsr*	-4.83
intE	5.64	*yafK*	1.14	*ymfM*	4.33	*fliF*	-5.2	*tyrR*	-1.11
iraP	1.46	*yafQ*	1.24	*ymfN*	4.16	*fliG*	-5.35	*ves*	-2.74
lamB	2.47	*yagK*	1.1	*ymfQ*	3.33	*fliH*	-5.08	*ycgR*	-4.96

lit	1.59	*yagL*	1.14	*ymfR*	3.51	*fliI*	-5.15	*yciT*	-2.06
Gene	**log_2FC**	**Gene**	**log_2FC**	**Gene**	**log_2FC**	**Gene**	**log_2FC**	**Gene**	**log_2FC**
livJ	3.18	*yahA*	2.59	*ymfS*	1.16	*fliJ*	-5.38	*yciZ*	-1.71
lrhA	1.2	*yahL*	1.17	*ymfT*	4.37	*fliK*	-4.84	*ycjF*	-1.3
lysU	3.38	*ybaJ*	1.44	*ymgA*	4.91	*fliL*	-5.47	*ycjQ*	-1.12
malK	2.88	*ybaS*	1.58	*ymgB*	4.16	*fliM*	-5.51	*ycjU*	-1.5
malM	2.4	*ybaT*	1.29	*ymgC*	3.54	*fliN*	-4.99	*ycjX*	-1.23
malP	1.57	*ybbC*	2.21	*ynbB*	1.27	*fliO*	-4.86	*ydcA*	-1.11
malQ	1.14	*ybcL*	1.71	*yoaC*	1.37	*fliP*	-4.89	*ydcM*	-1.16
matA	2.14	*ybcM*	1.88	*yoeB*	1.78	*fliQ*	-4.66	*yddA*	-3.21
mcrA	2.16	*ybcS*	1.03	*ypfM*	2.56	*fliR*	-3.34	*yddB*	-3.09
mdtE	1.36	*ybeD*	1.31	*yrbL*	1.06	*fliS*	-5.18	*ydeA*	-1.41
mdtF	1.57	*ybhQ*	1.48	*zntR*	1.55	*fliT*	-4.95	*ydeE*	-1.24
mokC	1.31	*ybiU*	1.08	*aceA*	-2.42	*fliZ*	-5.22	*ydfH*	-1.21
mqsR	4.15	*ybiV*	1.03	*aceB*	-2.5	*flxA*	-4.8	*ydfX*	-1.12
nrdA	1.26	*ycbW*	2.27	*aceK*	-2.25	*gcvH*	-2.46	*ydfZ*	-1.82
nrdB	1.43	*ycdT*	2.72	*adk*	-1.27	*gcvP*	-2.41	*ydgA*	-1.04
obgE	1.93	*ycdU*	2.83	*aroA*	-1.19	*gcvT*	-2.74	*ydgI*	-1.03
osmB	1.62	*yceJ*	1.72	*aroH*	-2.63	*gltB*	-5	*ydiE*	-2.23
pabC	1.47	*yceO*	1.51	*bglX*	-1.06	*gltD*	-4.65	*yecR*	-2.7
phoA	1.57	*ycfK*	1.63	*carA*	-2.79	*hisA*	-1.03	*yeiE*	-1.14
potE	1.11	*ycgZ*	3.68	*carB*	-2.43	*hisH*	-1.09	*ygfF*	-1.97
proV	1.62	*ydhY*	1.04	*cheA*	-5.78	*hmp*	-1.25	*yghJ*	-1.27
pspG	1.7	*ydjF*	1.42	*cheB*	-5.11	*htpG*	-1.3	*yhhJ*	-1.24
purC	1.19	*ydjH*	1.1	*cheR*	-5.34	*ilvH*	-1.57	*yhjG*	-1.17
qseB	1.64	*yeaI*	1.29	*cheW*	-6.09	*ilvI*	-1.81	*yhjH*	-4.97
qseC	1.03	*yebF*	2.41	*cheY*	-5.37	*leuA*	-1.73	*yjcZ*	-3.12
rbsD	1.87	*yebG*	2	*cheZ*	-5.13	*leuB*	-1.98	*yjdA*	-1.44
rcsA	1.75	*yebN*	1.96	*cirA*	-2.34	*leuC*	-1.93	*ykfB*	-1.11
recA	2.35	*yedW*	1.23	*codA*	-1.7	*leuD*	-1.76	*ymdA*	-2.5

Table S2: Confirmation of fold change obtained from microarray with qPCR [related to Figure 1]: Mean log_2FC of 3 DEGs viz. *folA*, *hdeA* and *gadX* in 4xR1, 4xR2, 32xR1 and 32xR2 obtained from microarray and qPCR (*rplF* and 16s as housekeeping controls). The qPCR was carried out using the same RNA that was used for microarray.

Gene	Type	4xR1	4xR2	32xR1	32xR2
folA	Microarray	2.85	3.87	4.7	3.78
folA	rplF	3.49	3.64	5	3.45
folA	16s	3.01	3.86	4.32	3.63
hdeA	Microarray	2.51	3.71	3.82	3.02
hdeA	rplF	2.8	3.44	4.47	3.14
hdeA	16s	2.31	3.65	3.79	3.32
gadX	Microarray	2.23	1.98	3.51	3.27
gadX	rplF	2.6	1.61	3.36	2.92
gadX	16s	2.11	1.82	2.67	3.09

Table S3: Selection of top-ranked shortest paths (top-paths) for 32xTopNet generation [related to Figure 2]: Shortest paths were sorted according to path cost and subsets of top-ranked shortest paths (top-paths) were analysed. DEG enrichment was estimated for different subsets. The number provided in bracket is the percentage of total genes (G=3435) or DEGs (D=345) that were picked in a particular subset. For topnet extraction, we sought a subset such that $d > 0.75*D$ and hypergeometric enrichment p-value ≤ 0.05. The hypergeometric probability is a measure of how many successes (DEGs-d) are included in a subset of the population (topnet-g) as compared to successes (D) present in the entire population (G). Subset containing top 0.4% top-ranked shortest paths (top-paths) was seen to satisfy these requirements.

% Top-paths	No. of Paths	Total no. of genes (g) (%)	DEGs (d) (%)	Enrichment p-value
0.05	4207	511 (15)	117 (34)	3.27E-22
0.1	8413	923 (27)	157 (45)	2.96E-16
0.15	12621	1478 (43)	203 (59)	3.66E-11
0.2	16828	2040 (59)	235 (68)	4.61E-05
0.25	21035	2172 (63)	240 (70)	0.001
0.3	25242	2308 (67)	248 (72)	0.007
0.35	29449	2415 (70)	252 (73)	0.047
0.4	**33656**	**2509 (73)**	**269 (78)**	**0.003**
0.45	37863	2863 (83)	292 (85)	0.086
0.5	42070	2961(86)	296 (86)	0.345

Table S4: : Confirmation of upregulation of genes in 32xR *E. coli* with qPCR [related to Figure 2]: (a) Normalized fold expression of: *glyA*, *csgD*, GASR (*gadA*, *gadB*, *gadE*) genes in WT grown in 0.125 µg/mL TMP, and 32xR1, 32xR2 and the clinical isolate (CI) grown in absence of TMP (b) *gcvT* in WT grown in 0.125 µg/mL TMP and CI grown in 16 µg/mL TMP; as compared to WT grown in the absence of TMP. Average of two replicates is shown (c) Primers and annealing temperatures.

(a) Normalized fold expression

Gene	WT-0.125 µg/mL TMP	32xR1	32xR2	CI
glyA	2.24	28.24	1.08	1.86
csgD	0.90	32.45	0.43	32.77
gadA	2.01	11.41	13.96	33.10
gadB	2.78	6.74	7.66	45.57
gadE	3.09	1.41	9.49	85.09

(b) Normalized fold expression

Gene	WT-0.125 µg/mL TMP	CI-16 µg/mL TMP
folA	3.42	2.14
gcvT	0.29	0.69
glyA	3.12	2.00

(c) Primers and annealing temperatures

Gene	Primer sequence (5'-3')		T_A (°C)
16s rRNA	FP	CGGACGGGTGAGTAATGTCT	58
	RP	CTCAGACCAGCTAGGGATCG	
glyA	FP	GGCTGGACGTTAGCGTAGTC	58
	RP	CTGATCGCCTCCGAAAACTA	
csgD	FP	CGATGAGTAAGGAGGGCTGA	58
	RP	TACCGCGACATTGAAAACTG	
gadA	FP	TTATGGACGTTTTCGTCGTC	55
	RP	GAAGCTGTTAACGGATTTCC	
gadB	FP	GCGGATTGCGGATATTCTTC	55
	RP	AGAATCAAAACGTTTTCCGC	
gadE	FP	TGGTAAACACTTGCCCCATAA	55
	RP	GTGACGATGTCGCTCATACG	
gcvT	FP	TGCCTCTGGCGGTGTGATAG	58
	RP	ACAGTGTGGCAGCTTTTGCC	
folA	FP	GATTGCGGCGTTAGCGGTAG	58
	RP	TTACGCGATCGTCCGTACCC	

Table S5: Generations completed after a particular number of hours by BW25113 and its *glyA* knockout [related to Figure 3]: It is seen that both strains complete similar number of generations after every 12 hours. Over a period of 14 days, ~180 generations are completed.

Hours	BW25113: mean	BW25113: SD	Δ*glyA*: mean	Δ*glyA*: SD
Dec-24	6.24	0.03	6.43	0.13
36	12.92	0.14	13.26	0.13
48	19.53	0.37	20.8	0.03
60	26.28	0.11	26.8	0.11
72	32.88	0.14	33.41	0.08
84	39.15	0.21	39.53	0.3
96	45.57	0.17	45.96	0.1
108	52.52	0.13	53.12	0.12
120	59.34	0.13	59.79	0.25
132	65.87	0.07	66.2	0.22
144	72.39	0.15	72.91	0.48
156	79.06	0.14	79.25	0.56
168	85.8	0.17	85.75	0.57
180	92.38	0.1	93.13	0.17
192	99.03	0.13	99.66	0.18
204	105.28	0.1	105.97	0.15
216	112.63	0.1	112.94	0.29
228	119.17	0.91	119.36	0.8
240	125.57	0.1	126.2	0.14
252	132.63	0.13	132.67	0.28
264	139.1	0.07	139.43	0.21
276	145.69	0.13	146.21	0.23
288	152.27	9.25	152.91	0.14
300	159.08	0.09	159.47	0.25
312	165.62	0.22	166.41	0.13
324	172.09	0.12	172.79	0.08
336	178.11	0.16	179.3	0.24

Transparent Methods

Strains, media, antibiotics and growth conditions: *E. coli* K12 MG1655 was used as the WT parent for evolution of 32xR (TMP-resistant) *E. coli*. Another K12 strain- *E. coli* BW25113 and BW25113:Δ*glyA* from the Keio collection, used for comparative evolution were purchased from the Coli Genetic Stock Centre, Yale University, New Haven, USA and revived using LB and LB-25 µg/mL kanamycin respectively as per instructions (Baba et al., 2006). The MDR-clinical isolate of uropathogenic *E. coli* was obtained from Ramaiah Memorial Hospital, Bangalore, India. All strains were grown in M9 minimal medium supplemented with 0.4% glucose and 0.4% Bacto™ casamino acids, at 37ºC and 180 rpm. The clinical isolate and 32xR *E. coli* were maintained in M9-16 µg/mL TMP to prevent loss of resistance. TMP (2 mg/mL), kanamycin (50 mg/mL) and chloramphenicol (35 mg/mL) were prepared in DMSO, distilled water and methanol respectively, filter sterilized and stored at -20ºC.

Minimum inhibitory concentration (MIC) measurement: Two-fold serial dilutions of TMP were prepared in a sterile 96- well plate in a final volume of 100 µL per well and inoculated with an appropriately diluted overnight culture such that each well contained ~5 x 10^5 cells. Estimation of cell density was carried out using freshly prepared McFarland's turbidity standard no. 0.5 (0.05 mL 1% $BaCl_2$ and 9.95 mL 1% H_2SO_4). The lowest concentration that visibly inhibited growth (A_{600} < 0.2) was noted as the MIC. Experiments were performed in triplicates.

Evolution of TMP-resistant (32xR) *E. coli*: Two well isolated colonies were selected and overnight cultures of the same were used to inoculate (1%) 20 mL M9 for WT controls and M9 with a sub-inhibitory concentration of TMP (0.125 µg/mL; 0.25 x MIC) for the evolution of resistant *E. coli*. Thus, a control and a resistant culture were derived from each colony. The TMP exposed cultures were allowed to attain an A_{600} ~ 0.6, following which they were used to inoculate the next batch of media containing a two-fold higher concentration of TMP, such that the initial A_{600} was at least 0.1. In all iterations thereafter, the TMP concentration was doubled until a concentration of 16 µg/mL (32 x MIC) was achieved. Adaptation beyond this concentration was not continued since it is likely to be outside the physiologically encountered range, as TMP is toxic to the host at a concentration of 20 µg/mL (Schulz and Schmoldt, 2003).

Microarray and transcriptome analysis

Samples: Cells were harvested from 40 mL exponential phase (A_{600} ~ 0.5) cultures of WT1, WT2, 4xR1, 4xR2, 32xR1 and 32xR *E. coli* at 5000 rpm for 10 minutes, snap frozen and stored at -80ºC. RNA was extracted using RNeasy Mini Kit (Qiagen). Quantification and estimation of purity with $A_{260/280}$ was done using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies). Integrity of RNA was verified on Agilent 2100 Bioanalyzer using RNA 6000 Nano LabChip (Agilent Technologies).

Labelling and hybridization: Labelling was performed using Quick-Amp Labelling Kit, One Colour Part Number 5190-0442 (Agilent Technologies), which employs T7 RNA polymerase which simultaneously amplifies target RNA and incorporates Cy3-labelled CTP. Hybridization of labelled RNA was done using Gene Expression Hybridization Kit (Agilent Technologies). A custom *E. coli* 8x15k array (AMADID: 019439) was used. RNA extraction, hybridization and data collection were done by Genotypic Technology Private Limited, Bangalore, India.

Transcriptome analysis: Raw data was processed using the limma package of R Bioconductor (Gentleman et al., 2004; Ritchie et al., 2015). Pre-processing included background correction, quantile normalization and filtering out of control and low expressing probes (R code in Supplementary Files). To filter out low expressing probes, 95th percentile of intensity values of all negative control probes on the array was calculated and probes expressing at least 15% brighter than this value were retained. Normalized signal intensity values for genes were obtained as corrected $\log_2$ transformed, probe averaged values of their respective raw signal intensities. Data fitting was performed using the linear modelling function "lmFit" in the limma package and a pairwise comparison between gene expression profiles of the three conditions was carried out to identify differentially expressed genes (DEGs): genes with $\log_2$Fold Change (FC) ≥ 1 (FDR-adjusted p-value < 0.05) between the WT and 4xR or 32xR *E. coli* were considered as DEGs. Gene enrichment analysis for DEGs was carried out using PANTHERv13 and the ClueGo v2.3 (Bindea et al., 2009; Mi et al., 2010).

***E. coli* protein-protein interaction network (EcPPIN) and 32xNet construction:** Base network/EcPPIN: Interactions between proteins in *E. coli* MG1655 were downloaded from STRING database v10 (Szklarczyk et al., 2015). STRING is a collection of direct (physical) and indirect (functional/regulatory) interactions between proteins observed through experiments or predicted (inferred) from bioinformatics methods based on domain fusion, phylogeny, gene co-expression and gene neighbourhood considerations. Each interaction in the database is associated with a confidence score on a scale of 0 to 1000 and interactions with score ≥ 700 are marked as "high-confidence". Only 19750 high-confidence interactions with a combined score ≥ 850 or experimental score ≥ 700 were selected. Mapping of gene names to b numbers (STRING v10 uses b numbers) was done using EcoGene 3.0 database (Zhou and Rudd, 2013). Finally, 19022 interactions between 3435 proteins for which we had gene expression data were retained for further analysis.

Several biological interactions are unidirectional and therefore, adding directions to a protein interaction network makes it biologically meaningful. Directions for regulatory interactions (TF → gene) were obtained from STRING v10, RegulonDB v7, EcoCyc and a study on organization of gene regulation in *E. coli* (Gama-Castro et al., 2011; Keseler et al., 2011; Shen-Orr et al., 2002; Szklarczyk et al., 2015). Directions for metabolic interactions were obtained from the *E. coli* genome scale metabolic reconstruction model iJO1366 using code developed earlier for extracting directed interactions between enzymes from a mathematical model (Asgari et al., 2013; Orth et al., 2011). Directions for interactions between genes encoding two component

systems were obtained from the KEGG database (Kanehisa and Goto, 2000). After a final round of manual curation, a high-confidence genome scale network, EcPPIN, containing 3498 genes and 24542 interactions of which 13631 (55.5%) were directed, was obtained.

32xNet: For 32xNet construction, weights were added to the genes (nodes) in EcPPIN i.e. it was made condition-specific to reflect transcriptomic differences between WT and 32xR *E. coli*. The node weight (NW) for a gene i in EcPPIN was the absolute log_2FC calculated as;

$$NW_i = |R_i - W_i|$$

where R_i and W_i are the fitted mean log_2 transformed signal intensities of gene i in 32xR (mean of 32xR1 and 32xR2) and WT (mean of WT1 and WT2) respectively.

Edge weight (EW_{ij}) for an interaction between genes i and j was calculated as;

$$EW_{ij} = NW_i \times NW_j$$

Shortest paths estimation and analysis of 32xTopNet: Inversed edge weight(s) (EW'_{ij}) for implementation of Dijkstra's algorithm were calculated as;

$$EW'_{ij} = (EW_{max}+EW_{min}) - EW_{ij};$$

where EW_{max} and EW_{min} are the maximum and minimum edge weights in the network. Finally, normalized path cost was calculated as

$$\text{Path cost} = (\Sigma EW'_{ij})/n$$

where n is the number of edges in the path.

Shortest paths were sorted(ranked) according to path cost and subsets (0.05% to 0.5% paths at an interval of 0.05%) containing top-ranked shortest paths (top-paths) were evaluated for DEG enrichment with hypergeometric test using SuperExactTest considering a total (n) of 3435 genes (Wang et al., 2015) (Table S3). Identification of clusters was done using ClusterONE in Cytoscape (Nepusz et al., 2012; Shannon et al., 2003).

Biofilm quantification

Crystal violet staining: WT was grown in 2 mL M9, M9-0.125 mg/L TMP and M9-0.25 mg/L TMP and 32xR strains were grown in 2 mL M9 and M9-16 mg/L TMP over a period of 5 days at room temperature without shaking in 24-well plates. Post incubation, the culture was decanted, the wells were gently washed with PBS and stained with 1% crystal violet for 15 minutes. Excess unbound dye was rinsed away with three distilled water washes. Quantification of the biofilm on the sides and the bottom of each well was done by dissolving the crystal violet with 2 mL absolute ethanol and recording the absorbance spectrophotometrically at 590 nm.

Scanning electron microscopy: The experiment was set up as described for the crystal violet staining with the addition of a sterile coverslip at the bottom of each well. Post incubation, the

culture was decanted, and the coverslips were transferred to clean wells, fixed with 2.5% glutaraldehyde for 24 hours at 4 ºC and washed with PBS post incubation. Serial dehydration was carried out using pre-chilled 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol. Vacuum desiccated coverslips were coated with gold for 38 seconds and images at 4000X, 8000X and 12000X were recorded using Thermo Scientific™ Quanta™ ESEM™ microscope.

Generation of *glyA* knockouts: Gene knockout was performed according to the protocol described elsewhere (Datsenko and Wanner, 2000). Briefly, *E. coli* was transformed with a plasmid pKD46 which has the red recombinase enzyme under the control of PBAD promoter, inducible by arabinose. Transformants harbouring pKD46 were grown in 5 mL of M9 containing ampicillin (50 µg/mL) and L-arabinose (20 mM) at 30°C. pKD3 was used for the amplification of the chloramphenicol resistance gene. Competent cells were transformed with the chloramphenicol resistance gene flanked by the homologous sequence of *glyA*. Transformants were selected on chloramphenicol (35 µg/mL) containing M9 plate. Putative knockout colonies were screened by a PCR based method with confirmatory primers and chloramphenicol resistance internal primers. The sequences of the primers used in this study are: 5'CTGTTATCGCACAATGATTCGGTTATACTGTTCGCCGTTGCATATGAATATCCTCCTTAG3' (Forward) and 5'ACATTGACAGCAAATCACCGTTTCGCTTATGCGTAAACCGGTGTAGGCTGGAGCTGCTTC3' (Reverse).

Comparative evolution: In a 96 well plate, two-fold dilutions of TMP were prepared ranging from 16 µg/mL to 0.125 µg/mL in a final volume of 100 µL and inoculated with 1 µL log phase cultures of BW25113:Δ*glyA* and its wild-type parent *E. coli* BW25113 obtained from 6 well isolated colonies of each strain. The plate was incubated at 37 ºC for 12 hours and 1 µL culture from the well with the highest TMP concentration showing an $A_{600} \geq A_{600}$ of the corresponding well without TMP (un-inhibited growth), was used to inoculate the next plate. Successive inoculations were carried out every 12 hours for 14 days. The generations completed in 12 hours for each replicate were calculated using a previously used formula (Zampieri et al., 2017): $\log_2(A_{600}\ (fin)/A_{600}(0)/100)$; where $A_{600}(fin)$ is the A_{600} obtained after 12 hours for a well X and $A_{600}(0)$ is the A_{600} of the well from which 1 µL of the culture was taken for inoculation of well X.

Supplemental references

Asgari, Y., Salehzadeh-Yazdi, A., Schreiber, F., and Masoudi-Nejad, A. (2013). Controllability in cancer metabolic networks according to drug targets as driver nodes. PLoS ONE *8*, e79397.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. *2*, 2006.0008.

Bindea, G., Mlecnik, B., Hackl, H., Charoentong, P., Tosolini, M., Kirilovsky, A., Fridman, W.-H., Pagès, F., Trajanoski, Z., and Galon, J. (2009). ClueGO: a Cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. Bioinformatics *25*, 1091–1093.

Datsenko, K.A., and Wanner, B.L. (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.

Gama-Castro, S., Salgado, H., Peralta-Gil, M., Santos-Zavaleta, A., Muñiz-Rascado, L., Solano-Lira, H., Jimenez-Jacinto, V., Weiss, V., García-Sotelo, J.S., López-Fuentes, A., et al. (2011). RegulonDB version 7.0: transcriptional regulation of Escherichia coli K-12 integrated within genetic sensory response units (Gensor Units). Nucleic Acids Res. *39*, D98-105.

Gentleman, R.C., Carey, V.J., Bates, D.M., Bolstad, B., Dettling, M., Dudoit, S., Ellis, B., Gautier, L., Ge, Y., Gentry, J., et al. (2004). Bioconductor: open software development for computational biology and bioinformatics. Genome Biol. *5*, R80.

Kanehisa, M., and Goto, S. (2000). KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids Res. *28*, 27–30.

Keseler, I.M., Collado-Vides, J., Santos-Zavaleta, A., Peralta-Gil, M., Gama-Castro, S., Muñiz-Rascado, L., Bonavides-Martinez, C., Paley, S., Krummenacker, M., Altman, T., et al. (2011). EcoCyc: a comprehensive database of Escherichia coli biology. Nucleic Acids Res. *39*, D583-590.

Mi, H., Dong, Q., Muruganujan, A., Gaudet, P., Lewis, S., and Thomas, P.D. (2010). PANTHER version 7: improved phylogenetic trees, orthologs and collaboration with the Gene Ontology Consortium. Nucleic Acids Res. *38*, D204-210.

Nepusz, T., Yu, H., and Paccanaro, A. (2012). Detecting overlapping protein complexes in protein-protein interaction networks. Nat. Methods *9*, 471–472.

Orth, J.D., Conrad, T.M., Na, J., Lerman, J.A., Nam, H., Feist, A.M., and Palsson, B.Ø. (2011). A comprehensive genome-scale reconstruction of Escherichia coli metabolism--2011. Mol. Syst. Biol. *7*, 535.

Ritchie, M.E., Phipson, B., Wu, D., Hu, Y., Law, C.W., Shi, W., and Smyth, G.K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res. *43*, e47.

Schulz, M., and Schmoldt, A. (2003). Therapeutic and toxic blood concentrations of more than 800 drugs and other xenobiotics. Pharmazie *58*, 447–474.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., Amin, N., Schwikowski, B., and Ideker, T. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. Genome Res. *13*, 2498–2504.

Shen-Orr, S.S., Milo, R., Mangan, S., and Alon, U. (2002). Network motifs in the transcriptional regulation network of Escherichia coli. Nat. Genet. *31*, 64–68.

Szklarczyk, D., Franceschini, A., Wyder, S., Forslund, K., Heller, D., Huerta-Cepas, J., Simonovic, M., Roth, A., Santos, A., Tsafou, K.P., et al. (2015). STRING v10: protein-protein interaction networks, integrated over the tree of life. Nucleic Acids Res. *43*, D447-452.

Wang, M., Zhao, Y., and Zhang, B. (2015). Efficient Test and Visualization of Multi-Set Intersections. Sci Rep *5*, 16923.

Zampieri, M., Enke, T., Chubukov, V., Ricci, V., Piddock, L., and Sauer, U. (2017). Metabolic constraints on the evolution of antibiotic resistance. Mol. Syst. Biol. *13*, 917.

Zhou, J., and Rudd, K.E. (2013). EcoGene 3.0. Nucleic Acids Res. *41*, D613-624.