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Supplemental Information

A Strategic Target Rescues

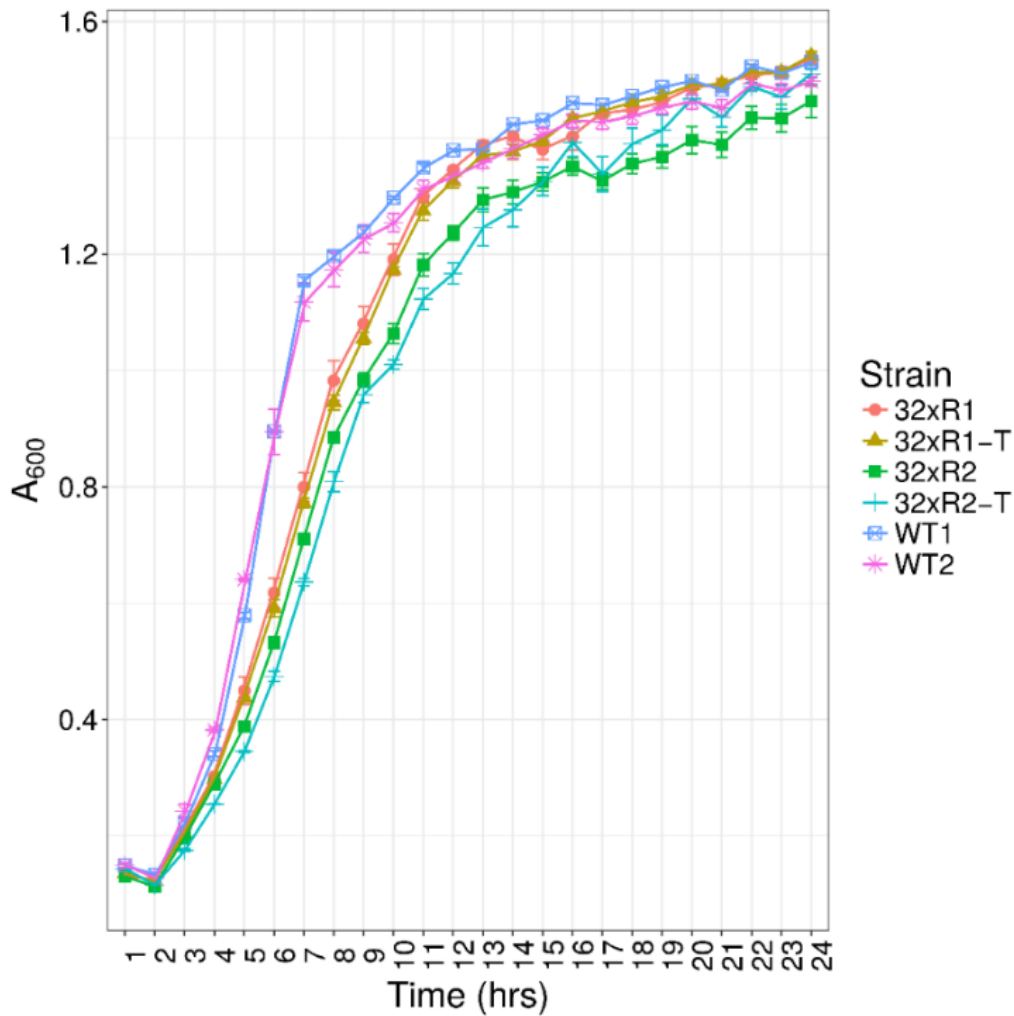
Trimethoprim Sensitivity in *Escherichia coli*

Amrisha Bhosle, Akshay Datey, Giridhar Chandrasekharan, Deepshikha Singh, Dipshikha Chakravortty, and Nagasuma Chandra

Supplementary Information

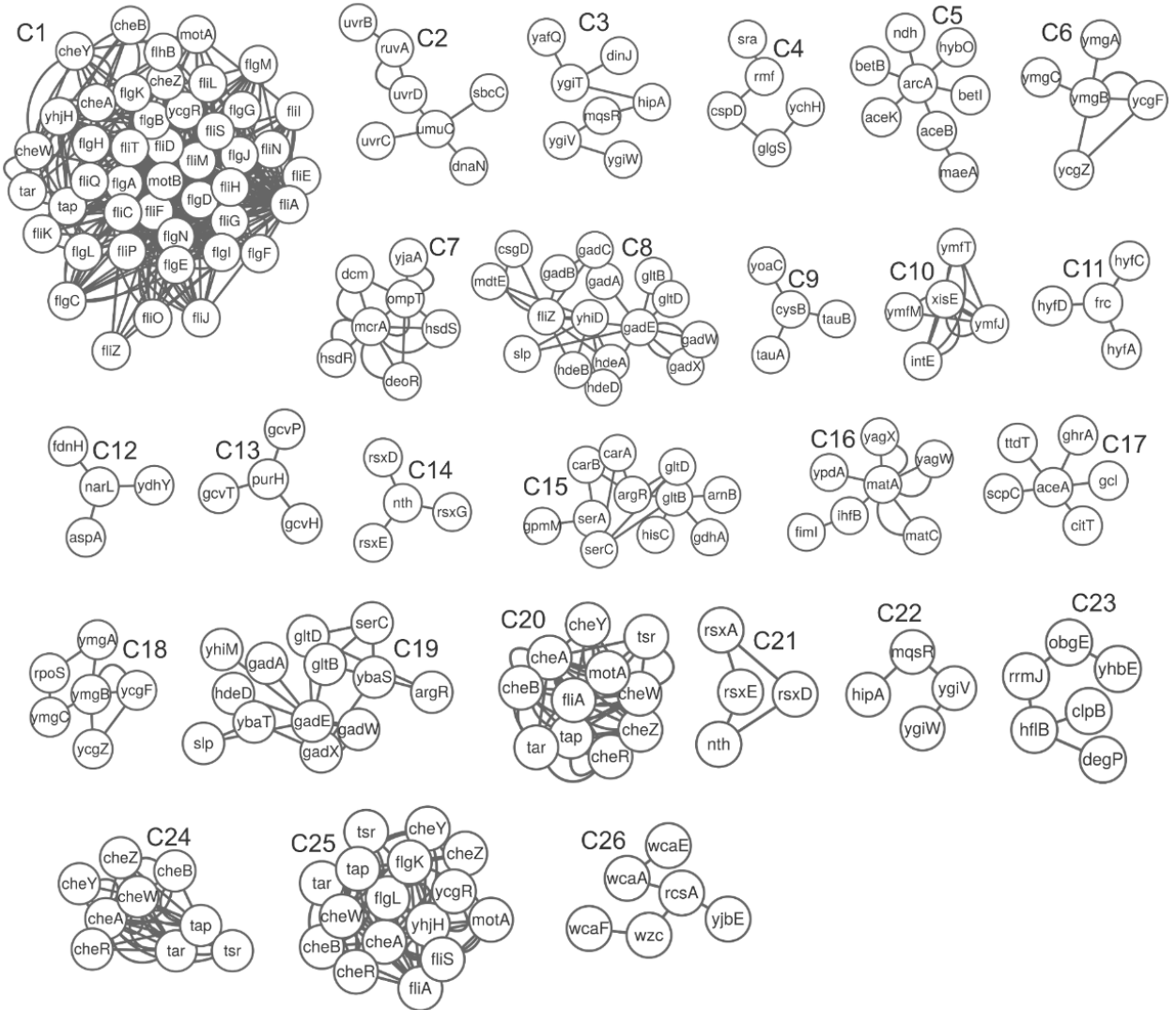
Supplementary Figures

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 $\mu\text{g}/\text{mL}$ TMP; and respective *E. coli* K12 MG1655 parents (WT1 and WT2) are shown. A_{600} recorded at each hour is shown as mean \pm 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 $\mu\text{g}/\text{mL}$ TMP.



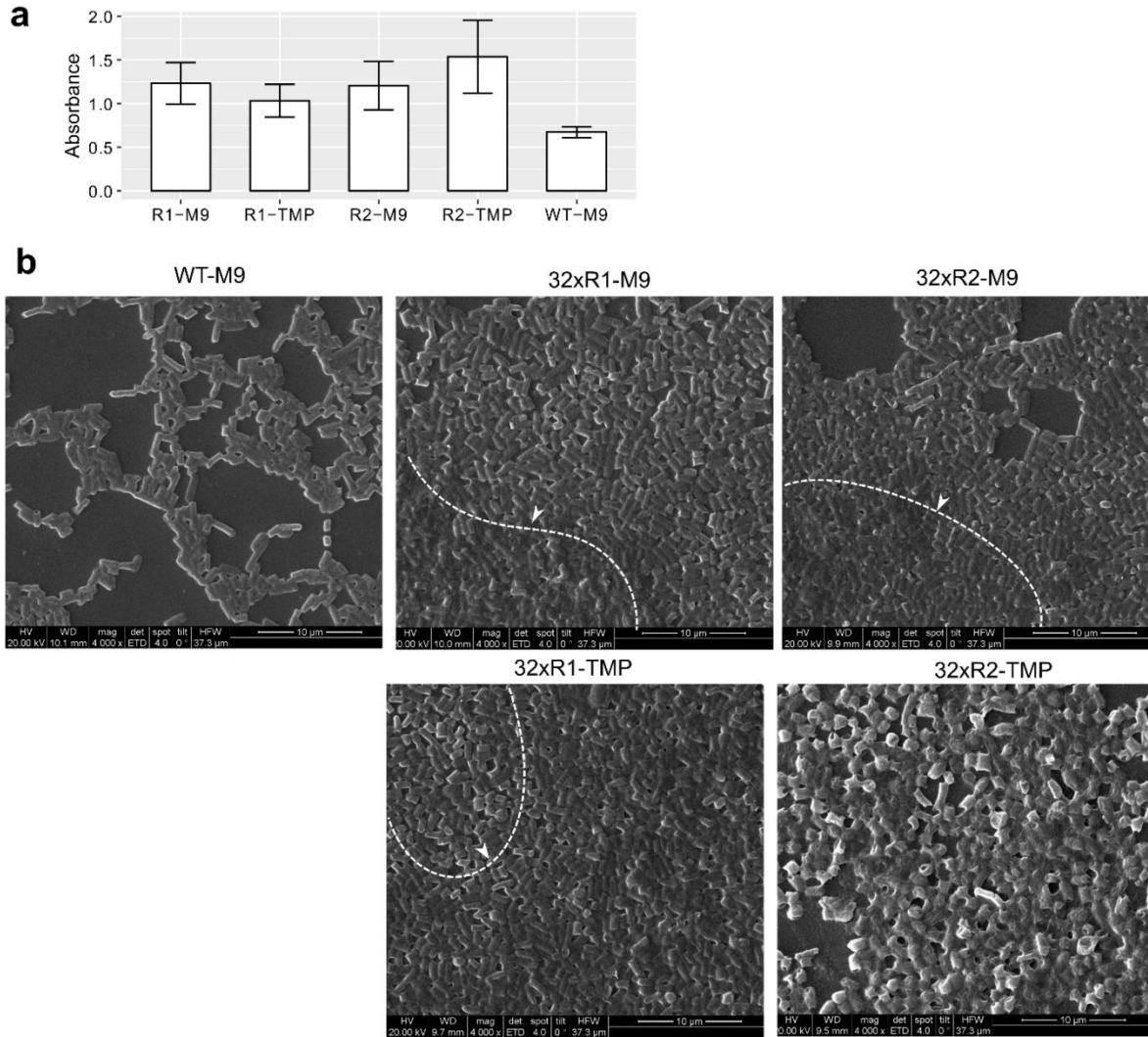
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16 **Figure S2: Clusters identified in 32xTopNet [related to Table 1]:** ClusterONE (Clustering
 17 with Overlapping Neighbourhood Expansion (Nepusz et al., 2012)) was used to identify
 18 clusters based on edge-weights. ClusterONE identifies clusters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



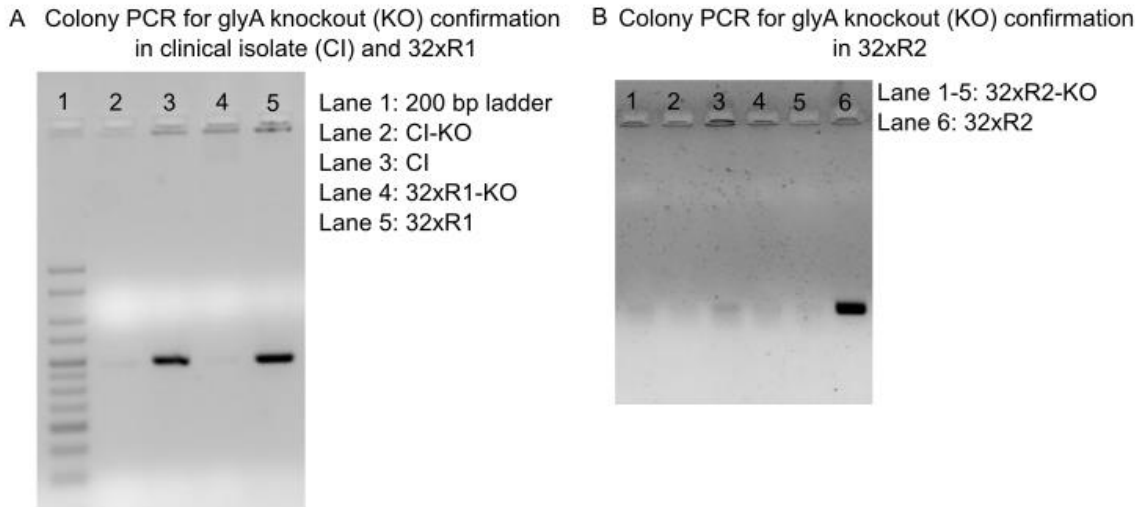
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31 **Figure S3: Biofilm formation [related to Table 1, Figure 1 and Figure 2]:** (a) Biofilm
 32 quantification by crystal violet staining (A_{590} data plotted as mean \pm SD) showed that biofilm
 33 production by the 32xR strains both in the absence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



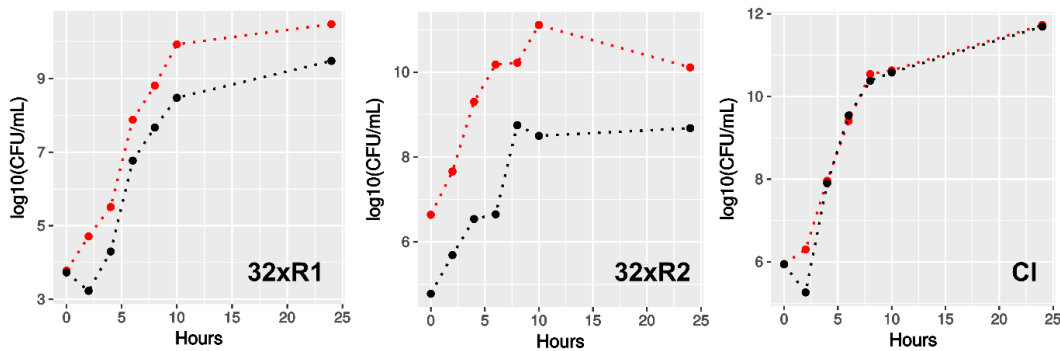
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46 **Figure S4: Confirmation of *glyA* knockouts [related to Figure 2C]**



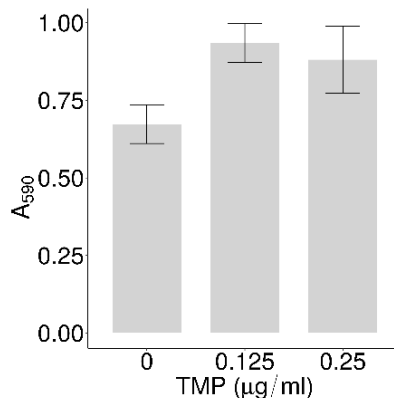
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48 **Figure S5: Growth curves of 32xR1, 32xR2 and CI and their respective *glyA* knockouts**
 49 **[related to Figure 2]:** Growth in the absence of TMP was profiled for 32xR strains and clinical
 50 isolate-CI (red) and their respective $\Delta glyA$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



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53 **Figure S6: Biofilm formation by WT in response to TMP stress [related to Figures 1 and**
 54 **Figure 3]:** Biofilm quantification by crystal violet staining (A_{590} data plotted as mean \pm SD)
 55 showed that biofilm production in WT increases upon exposure to sub-inhibitory, but stress
 56 inducing, TMP concentrations i.e. 0.25xMIC (0.125 $\mu\text{g/mL}$) and 0.5xMIC (0.25 $\mu\text{g/mL}$) (p-value
 57 < 0.01).



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59 **Supplementary Tables**

60 **Table S1: DEGs in the 32xR *E. coli* [related to Figure 1]:** log₂FC is the mean log₂FC for the
 61 32xR1 and 32xR2 strains

Gene	log ₂ FC	Gene	log ₂ FC	Gene	log ₂ FC	Gene	log ₂ FC	Gene	log ₂ FC
<i>ais</i>	1.81	<i>recN</i>	1.76	<i>yeeD</i>	2.55	<i>codB</i>	-1.99	<i>maeA</i>	-1.1
<i>allB</i>	1.61	<i>recX</i>	3.49	<i>yeeE</i>	2.88	<i>cyaA</i>	-1.21	<i>metE</i>	-2.01
<i>aphA</i>	1.41	<i>rfaB</i>	1.29	<i>yegJ</i>	1.33	<i>cybB</i>	-1.75	<i>mipA</i>	-1.06
<i>aspA</i>	2.82	<i>rfaI</i>	1.02	<i>yfaE</i>	1.18	<i>entD</i>	-1.24	<i>mntH</i>	-1.17
<i>betB</i>	1.87	<i>rfaS</i>	1.21	<i>yfbP</i>	1.57	<i>fdnH</i>	-1.19	<i>modA</i>	-1.73
<i>betI</i>	2.4	<i>rhsA</i>	1.32	<i>yfcV</i>	1.53	<i>fecR</i>	-1.18	<i>modB</i>	-1.63
<i>cadA</i>	1.02	<i>rhsD</i>	2.61	<i>yfdY</i>	1.21	<i>fepA</i>	-1.54	<i>modC</i>	-1.87
<i>csiE</i>	1.71	<i>ribB</i>	1.06	<i>ygck</i>	1.21	<i>fhuE</i>	-2.04	<i>modF</i>	-1.2
<i>dinG</i>	1.26	<i>rnf</i>	1.78	<i>yglL</i>	2.06	<i>fimA</i>	-5.72	<i>motA</i>	-4.91
<i>dinI</i>	2.13	<i>ruvA</i>	1.56	<i>ygcO</i>	1.03	<i>fimC</i>	-3.58	<i>motB</i>	-5.29
<i>dinJ</i>	1.33	<i>sbmC</i>	1.54	<i>ygdQ</i>	1.02	<i>fimD</i>	-2.42	<i>ndh</i>	-1.44
<i>dinQ</i>	2.83	<i>sfmD</i>	1.3	<i>ygiS</i>	2.9	<i>fimF</i>	-2.66	<i>ompF</i>	-1.35
<i>emrE</i>	1.35	<i>smpA</i>	1.06	<i>ygiT</i>	3.47	<i>fimG</i>	-2.62	<i>ompT</i>	-3.47
<i>fimB</i>	1.31	<i>sucA</i>	1.34	<i>ygiV</i>	1.24	<i>fimH</i>	-2.02	<i>oppA</i>	-1.17
<i>fimE</i>	1.21	<i>sulA</i>	3.57	<i>ygiW</i>	2.3	<i>fimI</i>	-4.58	<i>pntB</i>	-1.45
<i>folA</i>	4.27	<i>tauA</i>	1.16	<i>ygiZ</i>	1.02	<i>fiu</i>	-2.84	<i>pqqL</i>	-3.6
<i>frc</i>	1.1	<i>tauB</i>	1.01	<i>ygiN</i>	2.46	<i>flgA</i>	-4.89	<i>pyrB</i>	-3.06
<i>ftnB</i>	1.6	<i>tdcB</i>	1.33	<i>yhdN</i>	1.43	<i>flgB</i>	-5.87	<i>pyrD</i>	-1.53
<i>gadA</i>	2.82	<i>tfaE</i>	1.9	<i>yhhH</i>	1.42	<i>flgC</i>	-5.85	<i>pyrI</i>	-3
<i>gadB</i>	2.79	<i>tisA</i>	4.8	<i>yhiD</i>	3.02	<i>flgD</i>	-5.94	<i>rnb</i>	-1.11
<i>gadC</i>	2.15	<i>tisB</i>	5.07	<i>yhiM</i>	2.34	<i>flgE</i>	-5.55	<i>rsxD</i>	-1.1
<i>gadE</i>	3.55	<i>torA</i>	3.53	<i>yhiP</i>	2.35	<i>flgF</i>	-5.63	<i>rsxE</i>	-1.11
<i>gadX</i>	3.37	<i>torC</i>	5.91	<i>yhjX</i>	4.66	<i>flgG</i>	-5.31	<i>rsxG</i>	-1.06
<i>galE</i>	1.89	<i>torD</i>	3.09	<i>yibA</i>	1.17	<i>flgH</i>	-4.87	<i>sapA</i>	-1.33
<i>glgS</i>	1.33	<i>torY</i>	2.07	<i>yibD</i>	1.88	<i>flgI</i>	-4.69	<i>serA</i>	-3.03
<i>gltS</i>	1.42	<i>tyrP</i>	1.01	<i>yibT</i>	1.42	<i>flgJ</i>	-4.68	<i>shiA</i>	-1.18
<i>glyA</i>	1.26	<i>umuC</i>	3.95	<i>yibV</i>	2.22	<i>flgK</i>	-5.19	<i>speD</i>	-1.36
<i>guaA</i>	1.28	<i>umuD</i>	3.15	<i>yjbJ</i>	1.33	<i>flgL</i>	-4.7	<i>speE</i>	-1.26
<i>hdeA</i>	3.36	<i>wcaD</i>	1.58	<i>yjbM</i>	1.04	<i>flgM</i>	-4.73	<i>sufD</i>	-1.21
<i>hdeB</i>	3.14	<i>wcaE</i>	1.79	<i>yjbR</i>	1.46	<i>flgN</i>	-4.71	<i>tap</i>	-5.49
<i>hdeD</i>	2.74	<i>wcaF</i>	1.38	<i>yjeN</i>	1.48	<i>flhA</i>	-3.2	<i>tar</i>	-6.11
<i>hflB</i>	1.17	<i>xapR</i>	1.41	<i>yjfJ</i>	1.14	<i>flhB</i>	-3.99	<i>thrA</i>	-2.41
<i>hha</i>	1.14	<i>xisE</i>	5.66	<i>yjfK</i>	2.32	<i>flhE</i>	-3.66	<i>thrB</i>	-2.07
<i>hlyE</i>	1.28	<i>yacL</i>	1.51	<i>yjhl</i>	2.67	<i>fliA</i>	-5.4	<i>thrC</i>	-2.18
<i>htrL</i>	1.46	<i>yadC</i>	1.19	<i>ymfD</i>	1.9	<i>fliC</i>	-5.69	<i>trg</i>	-2.32
<i>hybO</i>	1.86	<i>yadI</i>	1.17	<i>ymfJ</i>	5.96	<i>fliD</i>	-5.47	<i>trpE</i>	-6.27
<i>idnD</i>	1.05	<i>yadK</i>	1.81	<i>ymfL</i>	4.8	<i>fliE</i>	-4.31	<i>tsr</i>	-4.83
<i>intE</i>	5.64	<i>yafK</i>	1.14	<i>ymfM</i>	4.33	<i>fliF</i>	-5.2	<i>tyrR</i>	-1.11
<i>iraP</i>	1.46	<i>yafQ</i>	1.24	<i>ymfN</i>	4.16	<i>fliG</i>	-5.35	<i>ves</i>	-2.74
<i>lamB</i>	2.47	<i>yagK</i>	1.1	<i>ymfQ</i>	3.33	<i>fliH</i>	-5.08	<i>ycgR</i>	-4.96

<i>lit</i>	1.59	<i>yagL</i>	1.14	<i>ymfR</i>	3.51	<i>fliI</i>	-5.15	<i>yciT</i>	-2.06
Gene	log₂FC	Gene	log₂FC	Gene	log₂FC	Gene	log₂FC	Gene	log₂FC
<i>livJ</i>	3.18	<i>yahA</i>	2.59	<i>ymfS</i>	1.16	<i>fliJ</i>	-5.38	<i>yciZ</i>	-1.71
<i>lrhA</i>	1.2	<i>yahL</i>	1.17	<i>ymfT</i>	4.37	<i>fliK</i>	-4.84	<i>ycjF</i>	-1.3
<i>lysU</i>	3.38	<i>ybaJ</i>	1.44	<i>ymgA</i>	4.91	<i>fliL</i>	-5.47	<i>ycjQ</i>	-1.12
<i>malk</i>	2.88	<i>ybaS</i>	1.58	<i>ymgB</i>	4.16	<i>fliM</i>	-5.51	<i>ycjU</i>	-1.5
<i>malM</i>	2.4	<i>ybaT</i>	1.29	<i>ymgC</i>	3.54	<i>fliN</i>	-4.99	<i>ycjX</i>	-1.23
<i>malP</i>	1.57	<i>ybbC</i>	2.21	<i>ynbB</i>	1.27	<i>fliO</i>	-4.86	<i>ydcA</i>	-1.11
<i>malQ</i>	1.14	<i>ybcL</i>	1.71	<i>yoaC</i>	1.37	<i>fliP</i>	-4.89	<i>ydcM</i>	-1.16
<i>matA</i>	2.14	<i>ybcM</i>	1.88	<i>yoeB</i>	1.78	<i>fliQ</i>	-4.66	<i>yddA</i>	-3.21
<i>mcrA</i>	2.16	<i>ybcS</i>	1.03	<i>ypfM</i>	2.56	<i>fliR</i>	-3.34	<i>yddb</i>	-3.09
<i>mdtE</i>	1.36	<i>ybeD</i>	1.31	<i>yrbL</i>	1.06	<i>fliS</i>	-5.18	<i>ydeA</i>	-1.41
<i>mdtF</i>	1.57	<i>ybhQ</i>	1.48	<i>zntR</i>	1.55	<i>fliT</i>	-4.95	<i>ydeE</i>	-1.24
<i>mokC</i>	1.31	<i>ybiU</i>	1.08	<i>aceA</i>	-2.42	<i>fliZ</i>	-5.22	<i>ydfH</i>	-1.21
<i>mqsR</i>	4.15	<i>ybiV</i>	1.03	<i>aceB</i>	-2.5	<i>flxA</i>	-4.8	<i>ydfX</i>	-1.12
<i>nrdA</i>	1.26	<i>ycbW</i>	2.27	<i>aceK</i>	-2.25	<i>gcvH</i>	-2.46	<i>ydfZ</i>	-1.82
<i>nrdB</i>	1.43	<i>ycdT</i>	2.72	<i>adk</i>	-1.27	<i>gcvP</i>	-2.41	<i>ydgA</i>	-1.04
<i>obgE</i>	1.93	<i>ycdU</i>	2.83	<i>aroA</i>	-1.19	<i>gcvT</i>	-2.74	<i>ydgl</i>	-1.03
<i>osmB</i>	1.62	<i>yceJ</i>	1.72	<i>aroH</i>	-2.63	<i>gltB</i>	-5	<i>ydiE</i>	-2.23
<i>pabC</i>	1.47	<i>yceO</i>	1.51	<i>bglX</i>	-1.06	<i>gltD</i>	-4.65	<i>yecR</i>	-2.7
<i>phoA</i>	1.57	<i>ycfK</i>	1.63	<i>carA</i>	-2.79	<i>hisA</i>	-1.03	<i>yeiE</i>	-1.14
<i>potE</i>	1.11	<i>ycgZ</i>	3.68	<i>carB</i>	-2.43	<i>hisH</i>	-1.09	<i>ygff</i>	-1.97
<i>proV</i>	1.62	<i>ydhY</i>	1.04	<i>cheA</i>	-5.78	<i>hmp</i>	-1.25	<i>yghJ</i>	-1.27
<i>pspG</i>	1.7	<i>ydjF</i>	1.42	<i>cheB</i>	-5.11	<i>htpG</i>	-1.3	<i>yhhJ</i>	-1.24
<i>purC</i>	1.19	<i>ydjH</i>	1.1	<i>cheR</i>	-5.34	<i>ilvH</i>	-1.57	<i>yhjG</i>	-1.17
<i>qseB</i>	1.64	<i>yeal</i>	1.29	<i>cheW</i>	-6.09	<i>ilvI</i>	-1.81	<i>yhjH</i>	-4.97
<i>qseC</i>	1.03	<i>yebF</i>	2.41	<i>cheY</i>	-5.37	<i>leuA</i>	-1.73	<i>yjcZ</i>	-3.12
<i>rbsD</i>	1.87	<i>yebG</i>	2	<i>cheZ</i>	-5.13	<i>leuB</i>	-1.98	<i>yjdA</i>	-1.44
<i>rcaA</i>	1.75	<i>yebN</i>	1.96	<i>cirA</i>	-2.34	<i>leuC</i>	-1.93	<i>ykfb</i>	-1.11
<i>recA</i>	2.35	<i>yedW</i>	1.23	<i>codA</i>	-1.7	<i>leuD</i>	-1.76	<i>ymdA</i>	-2.5

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72 **Table S2: Confirmation of fold change obtained from microarray with qPCR [related to**
 73 **Figure 1]:** Mean log₂FC of 3 DEGs viz. *folA*, *hdeA* and *gadX* in 4xR1, 4xR2, 32xR1 and 32xR2
 74 obtained from microarray and qPCR (*rplF* and 16s as housekeeping controls). The qPCR was
 75 carried out using the same RNA that was used for microarray.

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

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77 **Table S3: Selection of top-ranked shortest paths (top-paths) for 32xTopNet generation**
 78 **[related to Figure 2]:** Shortest paths were sorted according to path cost and subsets of top-
 79 ranked shortest paths (top-paths) were analysed. DEG enrichment was estimated for different
 80 subsets. The number provided in bracket is the percentage of total genes (G=3435) or DEGs
 81 (D=345) that were picked in a particular subset. For topnet extraction, we sought a subset
 82 such that $d > 0.75 * D$ and hypergeometric enrichment p-value ≤ 0.05 . The hypergeometric
 83 probability is a measure of how many successes (DEGs-d) are included in a subset of the
 84 population (topnet-g) as compared to successes (D) present in the entire population (G).
 85 Subset containing top 0.4% top-ranked shortest paths (top-paths) was seen to satisfy these
 86 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

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

98 **(a) Normalized fold expression**

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

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100 **(b) Normalized fold expression**

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

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105 **(c) Primers and annealing temperatures**

Gene	Primer sequence (5'-3')		T _A (°C)
<i>16s</i> <i>rRNA</i>	FP	CGGACGGGTGAGTAATGTCT	58
	RP	CTCAGACCAGCTAGGGATCG	
<i>glyA</i>	FP	GGCTGGACGTTAGCGTAGTC	58
	RP	CTGATCGCCTCCGAAAATA	
<i>csgD</i>	FP	CGATGAGTAAGGAGGGCTGA	58
	RP	TACCGCGACATTGAAAATA	
<i>gadA</i>	FP	TTATGGACGTTTTCGTCGTC	55
	RP	GAAGCTGTTAACGGATTTCC	
<i>gadB</i>	FP	GCGGATTGCGGATATTCTTC	55
	RP	AGAATCAAACGTTTTCCGC	
<i>gadE</i>	FP	TGGTAAACACTTGCCCCATAA	55
	RP	GTGACGATGTCGCTCATACG	
<i>gcvT</i>	FP	TGCCTCTGGCGGTGTGATAG	58
	RP	ACAGTGTGGCAGCTTTTGCC	
<i>folA</i>	FP	GATTGCGCGTTAGCGGTAG	58
	RP	TTACGCGATCGTCCGTACCC	

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

Hours	BW25113: mean	BW25113: SD	$\Delta glyA$: mean	$\Delta glyA$: 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

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121 **Transparent Methods**

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

133 **Minimum inhibitory concentration (MIC) measurement:** Two-fold serial dilutions of TMP
134 were prepared in a sterile 96- well plate in a final volume of 100 μ L per well and inoculated
135 with an appropriately diluted overnight culture such that each well contained $\sim 5 \times 10^5$ cells.
136 Estimation of cell density was carried out using freshly prepared McFarland's turbidity
137 standard no. 0.5 (0.05 mL 1% BaCl₂ and 9.95 mL 1% H₂SO₄). The lowest concentration that
138 visibly inhibited growth ($A_{600} < 0.2$) was noted as the MIC. Experiments were performed in
139 triplicates.

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

150 **Microarray and transcriptome analysis**

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

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

163 Transcriptome analysis: Raw data was processed using the limma package of R Bioconductor
164 (Gentleman et al., 2004; Ritchie et al., 2015). Pre-processing included background correction,
165 quantile normalization and filtering out of control and low expressing probes (R code in
166 Supplementary Files). To filter out low expressing probes, 95th percentile of intensity values of
167 all negative control probes on the array was calculated and probes expressing at least 15%
168 brighter than this value were retained. Normalized signal intensity values for genes were
169 obtained as corrected log₂ transformed, probe averaged values of their respective raw signal
170 intensities. Data fitting was performed using the linear modelling function “lmFit” in the limma
171 package and a pairwise comparison between gene expression profiles of the three conditions
172 was carried out to identify differentially expressed genes (DEGs): genes with log₂Fold Change
173 (FC) ≥ 1 (FDR-adjusted p-value < 0.05) between the WT and 4xR or 32xR *E. coli* were
174 considered as DEGs. Gene enrichment analysis for DEGs was carried out using PANTHERv13
175 and the ClueGo v2.3 (Bindea et al., 2009; Mi et al., 2010).

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

188 Several biological interactions are unidirectional and therefore, adding directions to a protein
189 interaction network makes it biologically meaningful. Directions for regulatory interactions (TF
190 → gene) were obtained from STRING v10, RegulonDB v7, EcoCyc and a study on organization
191 of gene regulation in *E. coli* (Gama-Castro et al., 2011; Keseler et al., 2011; Shen-Orr et al.,
192 2002; Szklarczyk et al., 2015). Directions for metabolic interactions were obtained from the *E.*
193 *coli* genome scale metabolic reconstruction model iJO1366 using code developed earlier for
194 extracting directed interactions between enzymes from a mathematical model (Asgari et al.,
195 2013; Orth et al., 2011). Directions for interactions between genes encoding two component

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

199 32xNet: For 32xNet construction, weights were added to the genes (nodes) in EcPPIN i.e. it
200 was made condition-specific to reflect transcriptomic differences between WT and 32xR *E.*
201 *coli*. The node weight (NW) for a gene *i* in EcPPIN was the absolute log₂FC calculated as;

$$202 \quad \mathbf{NW}_i = |R_i - W_i|$$

203 where R_i and W_i are the fitted mean log₂ transformed signal intensities of gene *i* in 32xR (mean
204 of 32xR1 and 32xR2) and WT (mean of WT1 and WT2) respectively.

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

$$206 \quad \mathbf{EW}_{ij} = \mathbf{NW}_i \times \mathbf{NW}_j$$

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

$$209 \quad \mathbf{EW}'_{ij} = (\mathbf{EW}_{\max} + \mathbf{EW}_{\min}) - \mathbf{EW}_{ij};$$

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

$$212 \quad \mathbf{Path \ cost} = (\Sigma \mathbf{EW}'_{ij}) / n$$

213 where *n* is the number of edges in the path.

214 Shortest paths were sorted(ranked) according to path cost and subsets (0.05% to 0.5% paths
215 at an interval of 0.05%) containing top-ranked shortest paths (top-paths) were evaluated for
216 DEG enrichment with hypergeometric test using SuperExactTest considering a total (*n*) of
217 3435 genes (Wang et al., 2015) (Table S3). Identification of clusters was done using
218 ClusterONE in Cytoscape (Nepusz et al., 2012; Shannon et al., 2003).

219 **Biofilm quantification**

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

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

230 culture was decanted, and the coverslips were transferred to clean wells, fixed with 2.5%
231 glutaraldehyde for 24 hours at 4 °C and washed with PBS post incubation. Serial dehydration
232 was carried out using pre-chilled 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol. Vacuum
233 desiccated coverslips were coated with gold for 38 seconds and images at 4000X, 8000X and
234 12000X were recorded using Thermo Scientific™ Quanta™ ESEM™ microscope.

235 **Generation of *glyA* knockouts:** Gene knockout was performed according to the protocol
236 described elsewhere (Datsenko and Wanner, 2000). Briefly, *E. coli* was transformed with a
237 plasmid pKD46 which has the red recombinase enzyme under the control of PBAD promoter,
238 inducible by arabinose. Transformants harbouring pKD46 were grown in 5 mL of M9
239 containing ampicillin (50 µg/mL) and L-arabinose (20 mM) at 30°C. pKD3 was used for the
240 amplification of the chloramphenicol resistance gene. Competent cells were transformed with
241 the chloramphenicol resistance gene flanked by the homologous sequence of *glyA*.
242 Transformants were selected on chloramphenicol (35 µg/mL) containing M9 plate. Putative
243 knockout colonies were screened by a PCR based method with confirmatory primers and
244 chloramphenicol resistance internal primers. The sequences of the primers used in this study
245 are: 5'CTGTTATCGACAATGATTCCGGTTATACTGTTCCGCGTTGCATATGAATATCCTCCTTAG3'
246 (Forward) and
247 5'ACATTGACAGCAAATCACCGTTTCGCTTATGCGTAAACCGGTGTAGGCTGGAGCTGCTTC3'
248 (Reverse).

249 **Comparative evolution:** In a 96 well plate, two-fold dilutions of TMP were prepared ranging
250 from 16 µg/mL to 0.125 µg/mL in a final volume of 100 µL and inoculated with 1 µL log phase
251 cultures of BW25113:Δ*glyA* and its wild-type parent *E. coli* BW25113 obtained from 6 well
252 isolated colonies of each strain. The plate was incubated at 37 °C for 12 hours and 1 µL culture
253 from the well with the highest TMP concentration showing an $A_{600} \geq A_{600}$ of the corresponding
254 well without TMP (un-inhibited growth), was used to inoculate the next plate. Successive
255 inoculations were carried out every 12 hours for 14 days. The generations completed in 12
256 hours for each replicate were calculated using a previously used formula (Zampieri et al.,
257 2017): $\log_2(A_{600}(\text{fin})/A_{600}(0)/100)$; where $A_{600}(\text{fin})$ is the A_{600} obtained after 12 hours for a well
258 X and $A_{600}(0)$ is the A_{600} of the well from which 1 µL of the culture was taken for inoculation of
259 well X.

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