# *In vivo* Titration of Folate Pathway Enzymes

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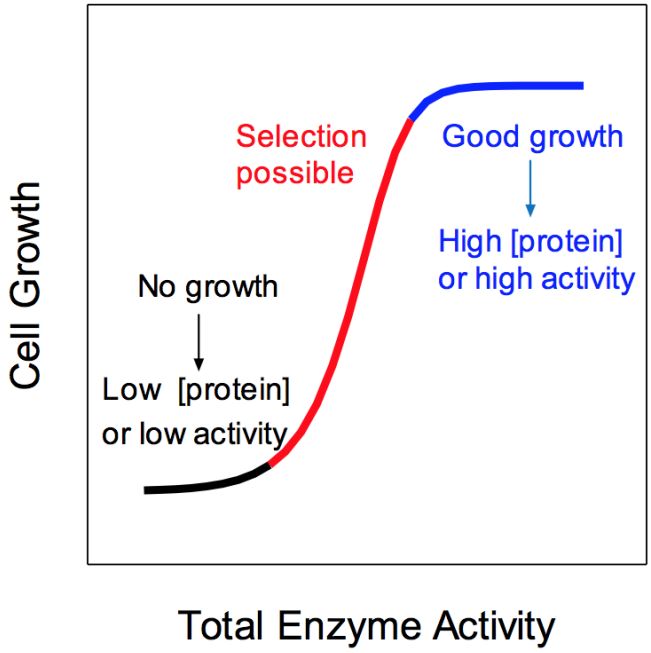



**Figure S1.** Effects of total enzyme activity on cell growth rate. The best selection occurs at the center of the graph (red line). The left-hand side of the graph (black line) describes conditions where the total enzyme activity is too low to enable growth. The right-hand side of the graph (blue line) describes conditions where selection is not possible due to high expression levels or high activity of the enzyme. Titration of enzyme activity is possible when either a low protein concentration or a low enzyme activity (due to mutation) occurs. Adapted from Butz et al. (1).

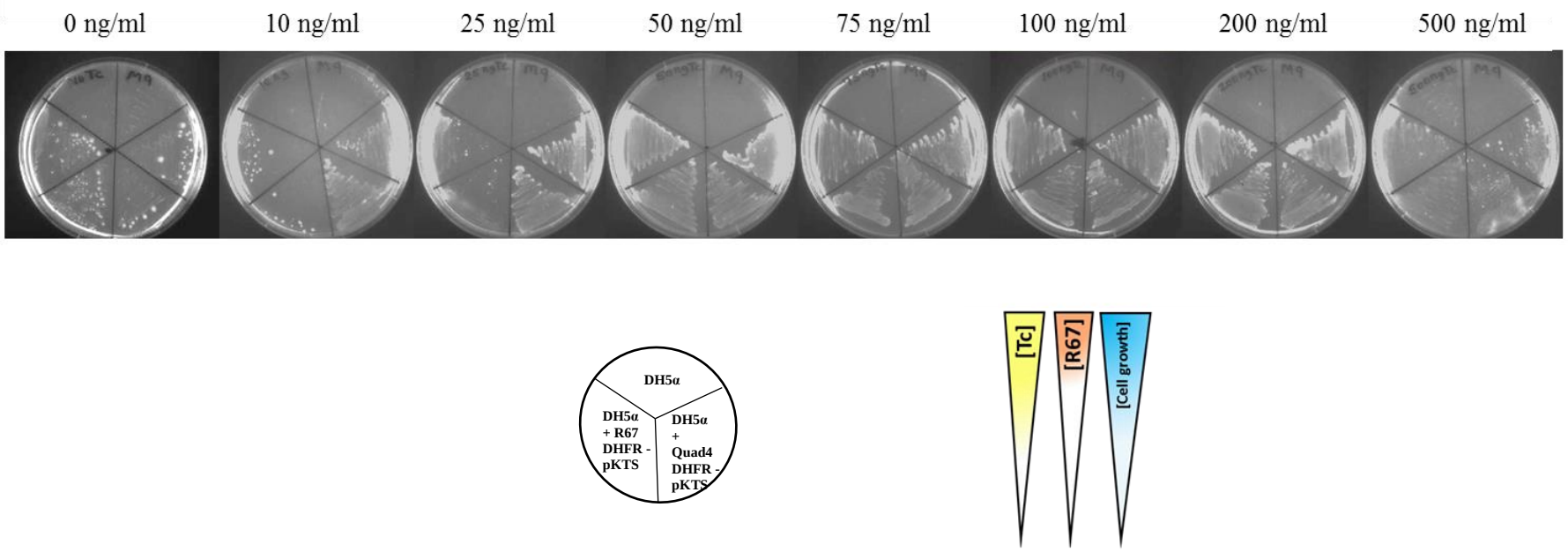



**Figure S2** shows a series of plates with increasing tetracycline concentration. DH5α cells carrying either no plasmid or R67 DHFR-pKTS or Quad4-pKTS were streaked on minimal M9 media containing 20 μg/ml TMP. Two colonies per plasmid transformant are shown. The tetracycline concentration is indicated above the plates. The tetracycline concentration required to produce sufficient DHFR activity for confluent cell growth was 50-200 ng/ml tetracycline for R67 DHFR-pKTS and 10-200 ng/ml for Quad4-pKTS. R67 DHFR is a homotetramer and will be tagged with four SsrA sequences as compared to Quad4 DHFR which will be a monomer with one SsrA tag. This will lead to increased degradation of R67 DHFR, thus more tetracycline is needed for the growth of these cells. The cartoon below shows how the constructs are streaked on the plates. The colored arrows indicate that with increasing tetracycline concentration (yellow), protein expression (red) is increased. This results in better cell growth (blue) until tetracycline begins acting as an antibiotic (2).

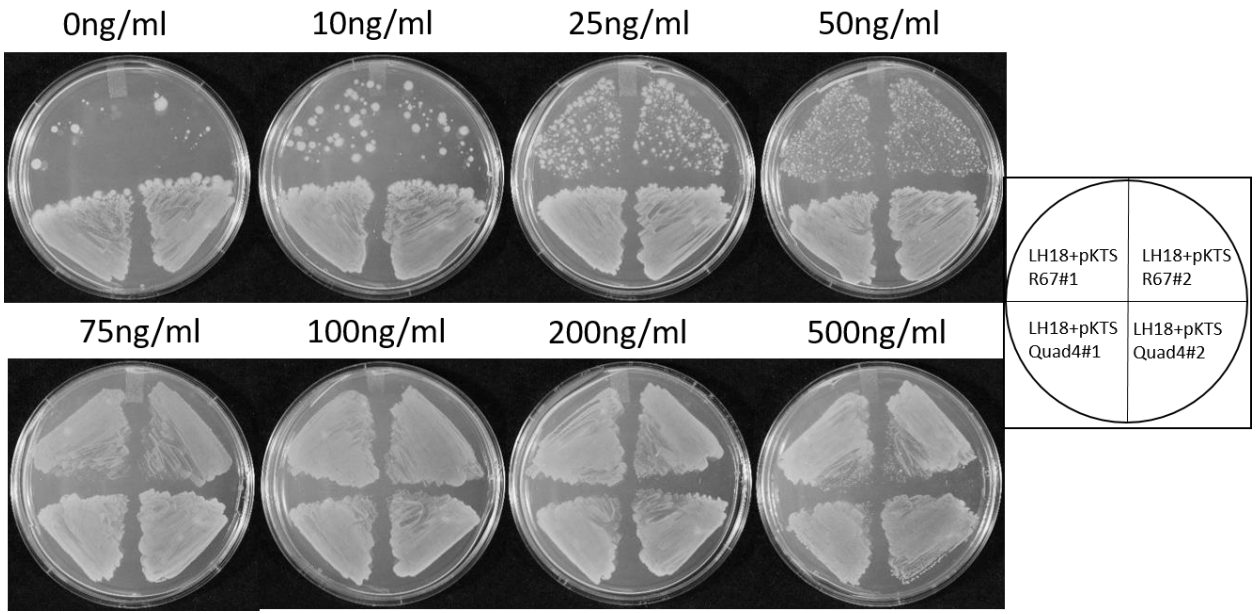



**Figure S3**: Growth of the LH18 Δ*folA* strains complemented by the R67 DHFR-pKTS or the Quad4-pKTS plasmids on minimal media in the presence of increasing concentrations of tetracycline after 5 days of incubation. The tetracycline concentration is indicated above the plates. Two clones of each complemented strain were streaked onto the plates. No growth at low tetracycline concentrations was seen for the first few days, with single colonies arising after ~2 days. Confluent growth was seen between 75-200ng/ml for R67 DHFR-pKTS, whereas good growth of Quad4-pKTS was seen on all concentrations of tetracycline.

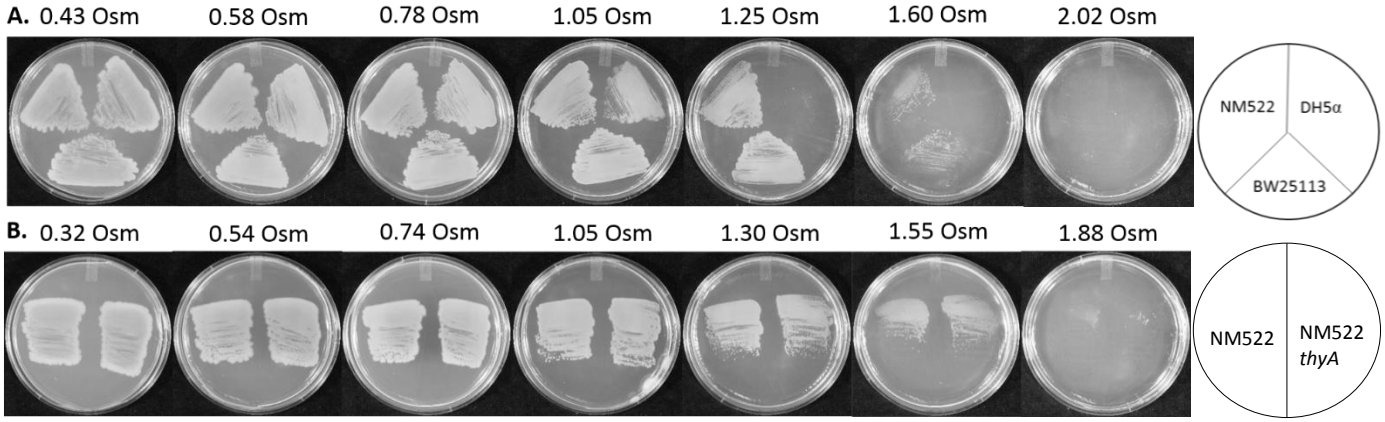



**Figure S4** shows how the parent *E. coli* strains are affected by osmotic stress when grown in Bonner Vogel minimal media with varying concentrations of sorbitol. NM522 and NM522 *thyA* are the parent strains for LH18 Δ*folA*. BW25113 is the parent strain for the Δ*metF* and Δ*glyA* Keio knockouts. Thymidine (200 μg/ml) was added to plates streaked by the NM522 *thyA* strain.

**Table S1.** Osmolalities at which growth stops on solid media in various *E. coli* strains.

| Strain | Media | Sorbitol (Osm) | NaCl (Osm) |
|---|---|---|---|
| DH5α | M9 minimal | $1.90 < x < 2.43$ | nd |
| DH5α + R67 DHFR-pKTS | M9 minimal + 20 μg/ml TMP | $0.81 < x < 1.28$ | nd |
| DH5α + Quad4-pKTS | M9 minimal + 20 μg/ml TMP | $1.60 < x < 1.93$ | nd |
| NM522 | BV minimal | $1.60 < x \leq 2.02$ | $1.6 < x < 1.8$ |
| NM522 *thyA* | BV minimal + thymidine | $1.55 < x < 1.88$ | $1.22 < x < 1.46$ |
| LH18 (Δ*folA*) | BV supplemented with thymidine, adenine, glycine, methionine, pantothenate | $1.24 < x < 1.52$ | nd |
| LH18 + R67 DHFR-pKTS | BV minimal + thymidine | $x \leq 0.57$ for mat growth; $0.57 < x \leq 0.88$ range for overgrowing colonies | nd |
| LH18 + Quad4-pKTS | BV minimal + thymidine | $1.15 < x < 1.81$ | nd |
| LH18 (Δ*folA*) | BV supplemented with thymidine, adenine, glycine, methionine, pantothenate + 1 mM betaine | $1.81 < x < 2.22$ | nd |
| LH18 + R67 DHFR-pKTS | BV minimal + thymidine + 1 mM betaine | $x < 0.71$ for mat growth; $1.40 < x < 1.67$ range for overgrowing colonies | nd |
| LH18 + Quad4-pKTS | BV minimal + thymidine + 1 mM betaine | $1.97 < x < 2.15$ | nd |
| BW25113 | BV minimal | $1.60 < x \leq 2.02$ | $1.6 < x < 1.8$ |
| Δ*metF* | BV minimal + methionine | $1.64 < x < 2.21$ | $1.35 < x \leq 1.44$ |
| Δ*metF* + MTHFR-pKTS | BV minimal | $x \leq 1.20$ for mat growth; $1.20 < x < 1.67$ range for overgrowing colonies | $x < 0.48$ for mat growth; $0.48 < x \leq 0.71$ (overgrowing colonies) |
| Δ*glyA* | BV minimal + glycine + serine | $1.38 < x < 1.71$ | $1.15 < x \leq 1.33$ |
| Δ*glyA* + SHMT-pKTS | BV minimal | $0.92 \leq x$ for mat growth; $0.92 < x < 1.30$ (overgrowing colonies) | $x < 0.53$ for mat growth; $0.53 < x \leq 0.70$ (overgrowing colonies) |
| KA12/pKIMP-UAUC | BV minimal + phenylalanine + tyrosine | $1.35 < x < 1.82$ | nd |
| KA12/pKIMP-UAUC + CM-pKTS | BV minimal | $0.65 \leq x$ for mat growth; $0.65 < x < 1.40$ (overgrowing colonies) | nd |

**Table S2.** Doubling times for the various *E. coli* strains in minimal BV liquid media. Mutant strains had the appropriate supplements added. Growth curves were performed in triplicate. Tetracycline concentrations used are listed in Table 5.

| **Strain** | **Osmolality of sorbitol containing media (Osm)** | **Doubling time in sorbitol containing media (mins)** | **Osmolality of NaCl containing media (Osm)** | **Doubling Time in NaCl containing media (mins)** |
|---|---|---|---|---|
| NM522 | 0.18 | 130 ± 10 | 0.18 | 210 ± 10 |
| | 0.44 | 160 ± 20 | 0.37 | 270 ± 10 |
| | 0.67 | 180 ± 20 | 0.51 | 950 ± 20 |
| | 0.93 | 340 ± 10 | 0.70 | 2100 ± 20 |
| | 1.07 | 1100 ± 30 | 0.88 | 3500 ± 90 |
| | 1.25 | 0 | 1.08 | 0 |
| | | | | |
| NM522 *thyA* | 0.18 | 120 ± 10 | 0.18 | 170 ± 5 |
| | 0.44 | 100 ± 20 | 0.37 | 160 ± 5 |
| | 0.67 | 100 ± 20 | 0.51 | 350 ± 10 |
| | 0.93 | 470 ± 30 | 0.70 | 3500 ± 200 |
| | 1.07 | 1200 ± 50 | 0.88 | 6900 ± 300 |
| | 1.25 | 0 | 1.08 | 0 |
| | | | | |
| LH18= | 0.22 | 200 ± 10 | 0.19 | 250 ± 10 |
| NM522 *thyA* | 0.52 | 240 ± 10 | 0.35 | 170 ± 10 |
| Δ*folA::kan* | 0.67 | 360 ± 20 | 0.55 | 490 ± 20 |
| | 0.84 | 1100 ± 20 | 0.60 | 900 ± 20 |
| | 1.08 | 3500 ± 70 | 0.81 | 1200 ± 60 |
| | 1.16 | 0 | 1.04 | 1700 ± 40 |
| | | | 1.12 | 1800 ± 100 |
| | | | 1.4 | 0 |
| | | | | |
| LH18Δ*folA* | 0.22 | 260 ± 10 | 0.19 | 220 ± 10 |
| + R67 DHFR- | 0.52 | 280 ± 20 | 0.35 | 520 ± 20 |
| pKTS | 0.67 | 900 ± 25 | 0.55 | 1500 ± 140 |
| | 0.84 | 0 | 0.60 | 0 |
| | | | | |
| LH18Δ*folA* | 0.22 | 220 ± 10 | 0.19 | 280 ± 10 |
| + Quad4 DHFR- | 0.52 | 220 ± 30 | 0.35 | 490 ± 20 |
| pKTS | 0.67 | 540 ± 40 | 0.55 | 1200 ± 80 |
| | 0.84 | 2400 ± 60 | 0.60 | 1700 ± 100 |
| | 1.08 | 2400 ± 30 | 0.81 | 1800 ± 40 |
| | 1.16 | 7200 ± 200 | 1.04 | 1900 ± 40 |
| | 1.42 | 0 | 1.12 | 2300 ± 50 |
| | | | 1.4 | 0 |
| | | | | |
| BW25113 | 0.21 | 110 ± 5 | 0.21 | 120 ± 5 |
| | 0.47 | 150 ± 5 | 0.35 | 140 ± 10 |
| | 0.67 | 230 ± 10 | 0.54 | 140 ± 10 |

| | | | | |
|---|---|---|---|---|
| | 0.88 | 280 ± 10 | 0.65 | 170 ± 5 |
| | 1.08 | 360 ± 20 | 0.82 | 230 ± 20 |
| | 1.28 | 3500 ± 90 | 1.05 | 240 ± 10 |
| | 1.53 | 0 | 1.13 | 780 ± 20 |
| | | | | |
| Δ*metF* | 0.18 | 130 ± 5 | 0.19 | 140 ± 5 |
| | 0.37 | 150 ± 5 | 0.33 | 130 ± 5 |
| | 0.6 | 160 ± 5 | 0.53 | 140 ± 5 |
| | 0.86 | 220 ± 20 | 0.73 | 190 ± 5 |
| | 1.07 | 380 ± 20 | 0.79 | 250 ± 10 |
| | 1.25 | 870 ± 70 | 0.98 | 460 ± 20 |
| | 1.47 | 0 | 1.18 | 2200 ± 70 |
| | | | 1.47 | 0 |
| | | | | |
| Δ*metF* + MTHFR-pKTS | 0.18 | 290 ± 5 | 0.19 | 300 ±10 |
| | 0.37 | 610 ± 20 | 0.33 | 530 ± 10 |
| | 0.6 | 1400 ± 20 | 0.53 | 860 ± 10 |
| | 0.86 | 2100 ± 40 | 0.73 | 950 ± 10 |
| | 1.07 | 2300 ± 40 | 0.79 | 1400 ± 20 |
| | 1.25 | 4600 ± 50 | 0.98 | 1700 ± 60 |
| | 1.47 | 0 | 1.18 | 2300 ± 60 |
| | | | 1.47 | 0 |
| | | | | |
| Δ*glyA* | 0.18 | 150 ± 5 | 0.18 | 120 ± 5 |
| | 0.47 | 170 ± 5 | 0.39 | 120 ± 5 |
| | 0.67 | 170 ± 5 | 0.54 | 170 ± 5 |
| | 0.93 | 180 ± 5 | 0.66 | 170 ± 5 |
| | 1.11 | 350 ± 10 | 0.86 | 200 ± 5 |
| | 1.29 | 0 | 1.05 | 480 ± 50 |
| | | | 1.20 | 750 ± 50 |
| | | | 1.31 | 0 |
| | | | | |
| Δ*glyA* + SHMT-pKTS | 0.18 | 390 ±20 | 0.18 | 240±10 |
| | 0.47 | 800 ± 40 | 0.39 | 500 ± 10 |
| | 0.67 | 990 ± 20 | 0.54 | 1000 ± 10 |
| | 0.93 | 1400 ± 20 | 0.66 | 2700 ± 50 |
| | 1.11 | 3100 ± 200 | 0.86 | 6900 ± 300 |
| | 1.29 | 0 | 1.05 | 0 |

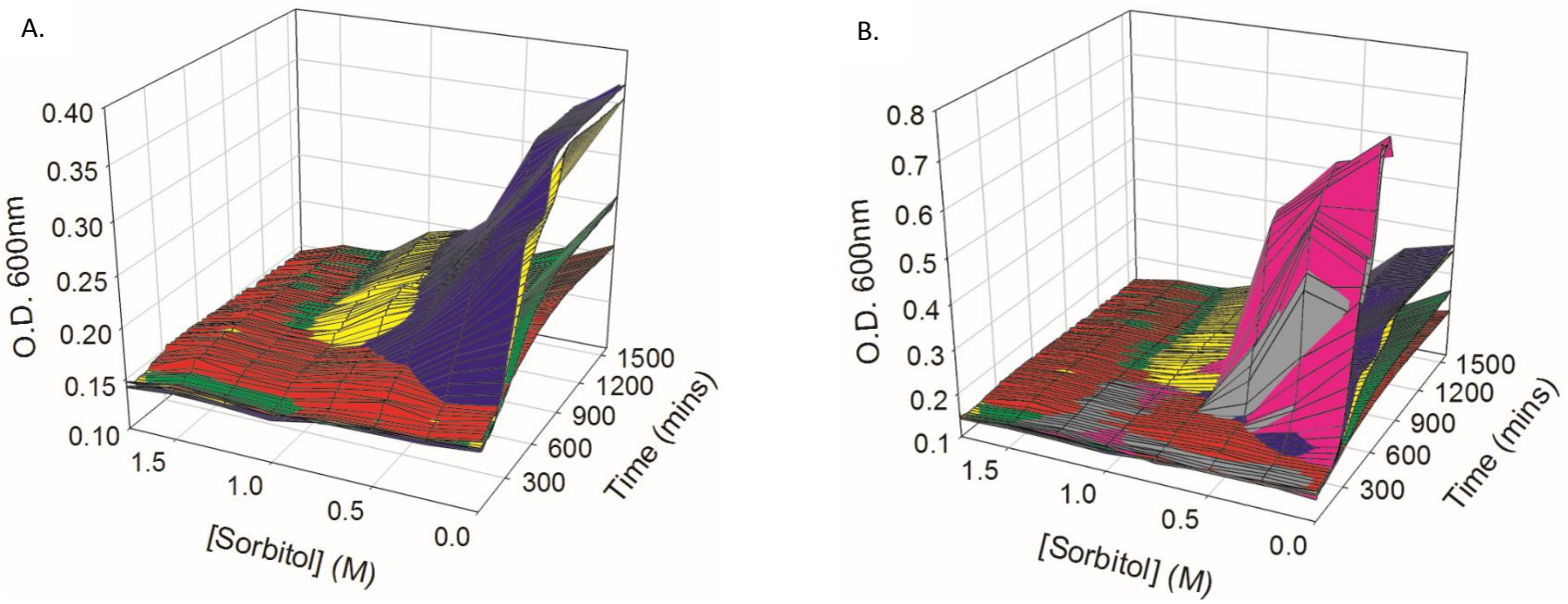



**Figure S5** provides 3D plots of cell growth. Left panel (A): growth in minimal media for the LH18 Δ*folA* strain complemented by the R67 DHFR-pKTS plasmid. Tetracycline concentrations are 0 ng/ml (red), 50 ng/ml (green), 100 ng/ml (yellow) and 200ng/ml (blue). Sorbitol concentrations are shown on the x-axis. Right panel, the same data with the growth curves from the parent strains NM522 (magenta) and NM522*thyA8* (gray) added. The faster growth of the parent strains is consistent with the Hilvert group's proposal that the pKTS plasmid results in protein concentrations/activity that are lower than those encoded by the chromosome (3). Neuenschwander et al. supported their hypothesis by monitoring GFP expression in *E. coli* (3).

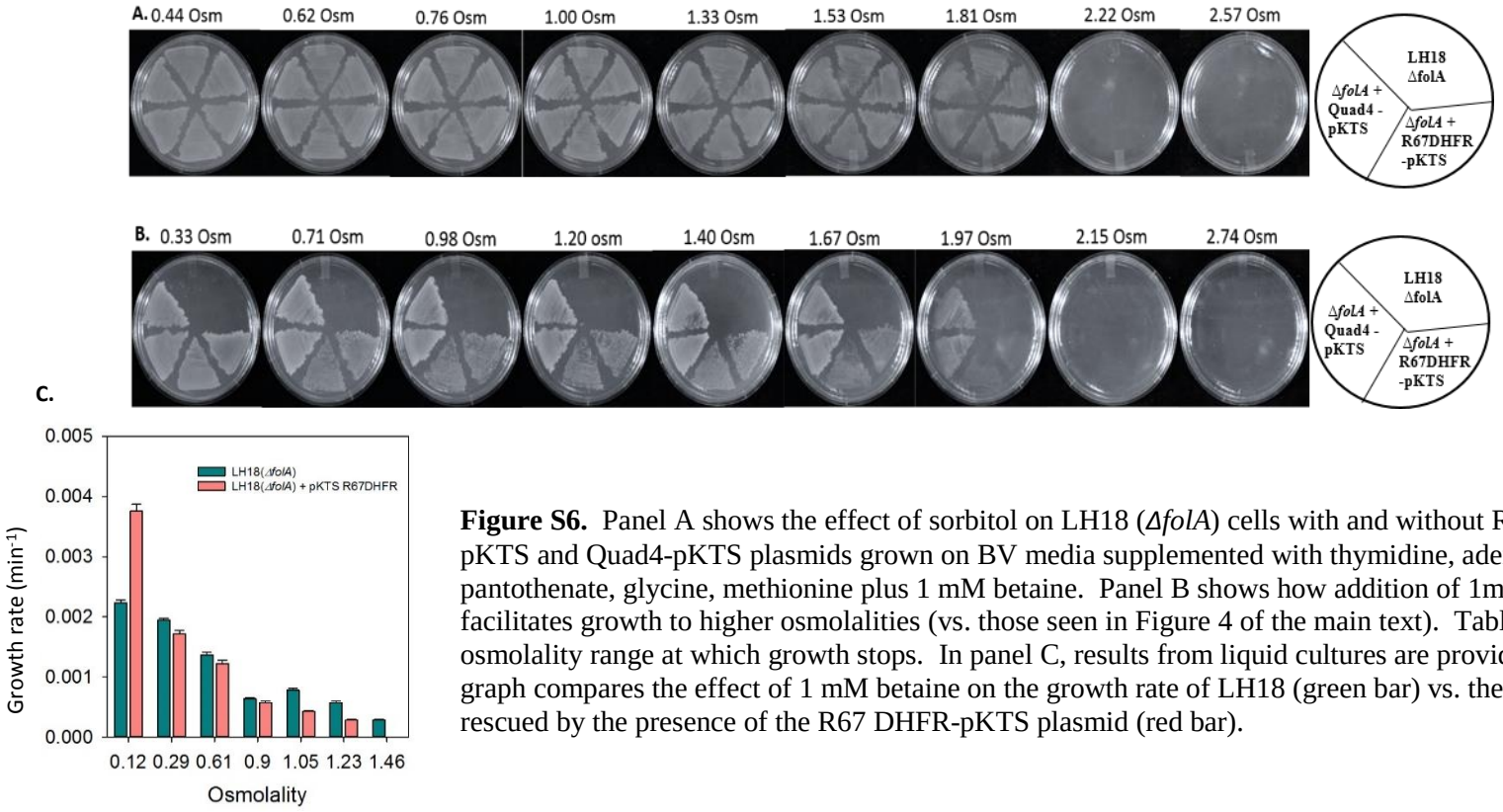



**Figure S6.** Panel A shows the effect of sorbitol on LH18 (*ΔfolA*) cells with and without R67 DHFR-pKTS and Quad4-pKTS plasmids grown on BV media supplemented with thymidine, adenine, pantothenate, glycine, methionine plus 1 mM betaine. Panel B shows how addition of 1mM betaine facilitates growth to higher osmolalities (vs. those seen in Figure 4 of the main text). Table S1 lists the osmolality range at which growth stops. In panel C, results from liquid cultures are provided. The bar graph compares the effect of 1 mM betaine on the growth rate of LH18 (green bar) vs. the cells rescued by the presence of the R67 DHFR-pKTS plasmid (red bar).

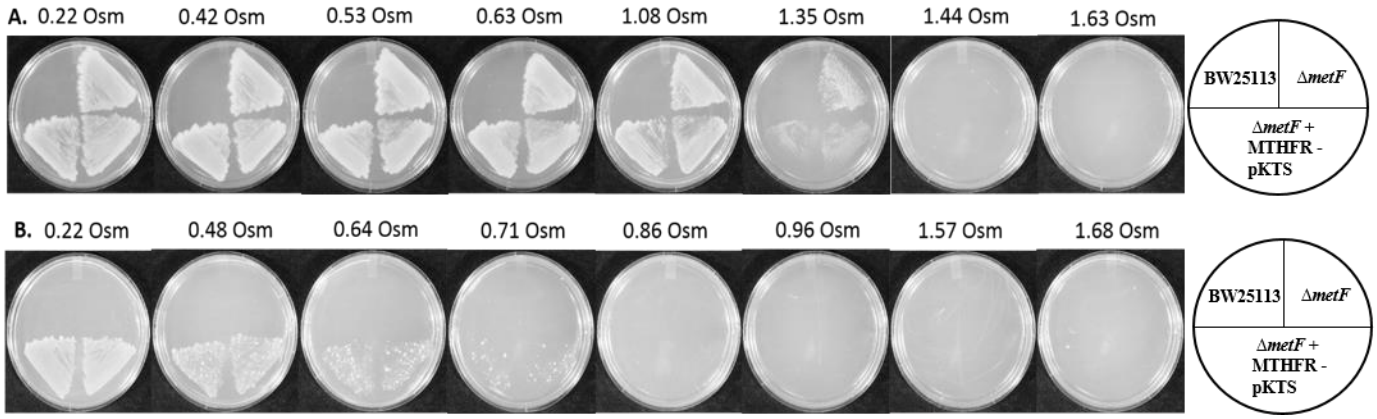



**Figure S7.** Osmotic stress titrations of Δ*metF* strains using NaCl. Panel A (top) shows the growth of deletion and complemented strains in BV media supplemented with methionine and kanamycin. The cells grow until $1.35 < x < 1.44$ Osm. Panel B (bottom) shows the growth in BV minimal media with kanamycin, ampicillin and 100ng/ml tetracycline. Complemented cells carrying the MTHFR-pKTS plasmid grew confluently until 0.48 Osm. Isolated overgrowing colonies were observed until 0.71 Osm. The cartoons on the right provide a template for the positions of the different strains.

**A.** **B.**

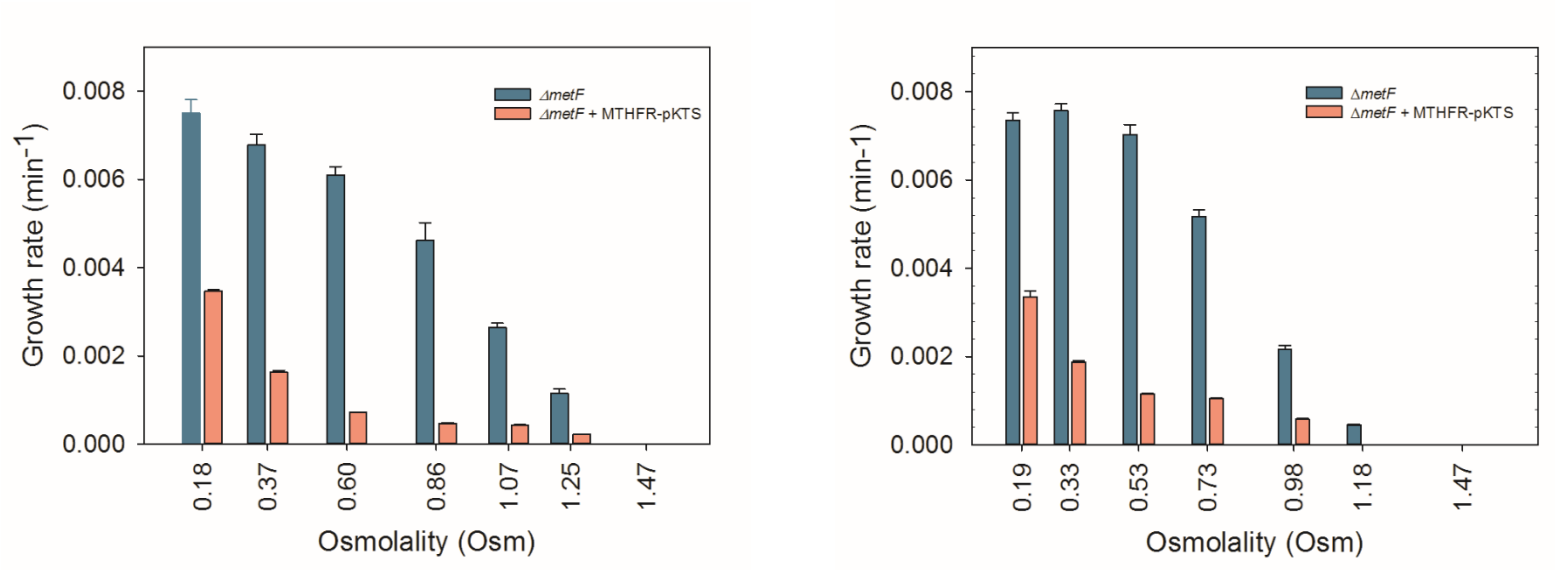



**Figure S8.** Plots of growth rates ($min^{-1}$) vs. osmolality for the Δ*metF* strain. Panel A (left) shows growth in the presence of sorbitol for the deletant strain (green bar) and the complemented strain (red bar). Panel B (right) shows growth in the presence of NaCl. With both osmotic stressors, the deletion strains were grown in BV media supplemented with methionine whereas the complemented strains (Δ*metF*+MTHFR-pKTS) were grown in minimal media with 75ng/ml tetracycline.

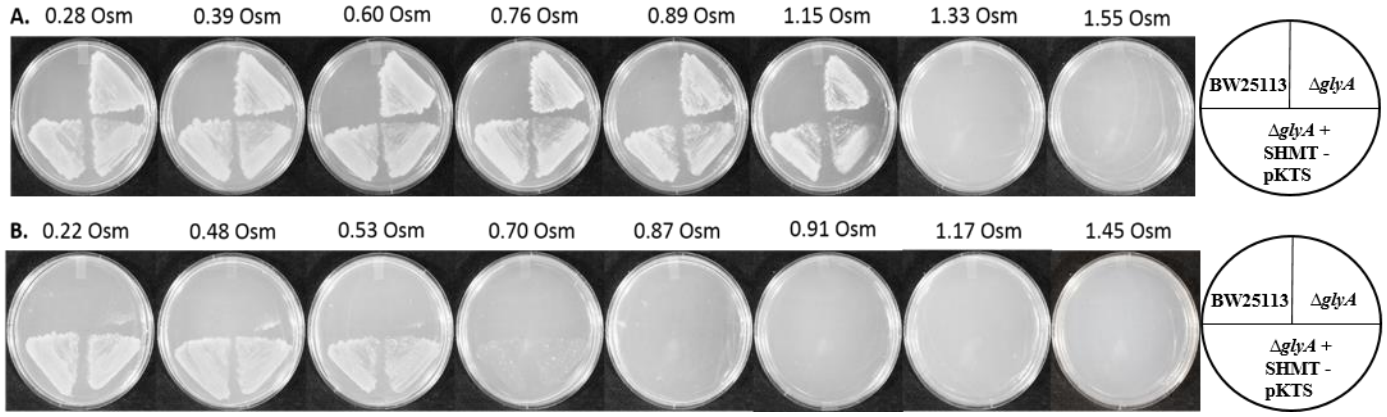
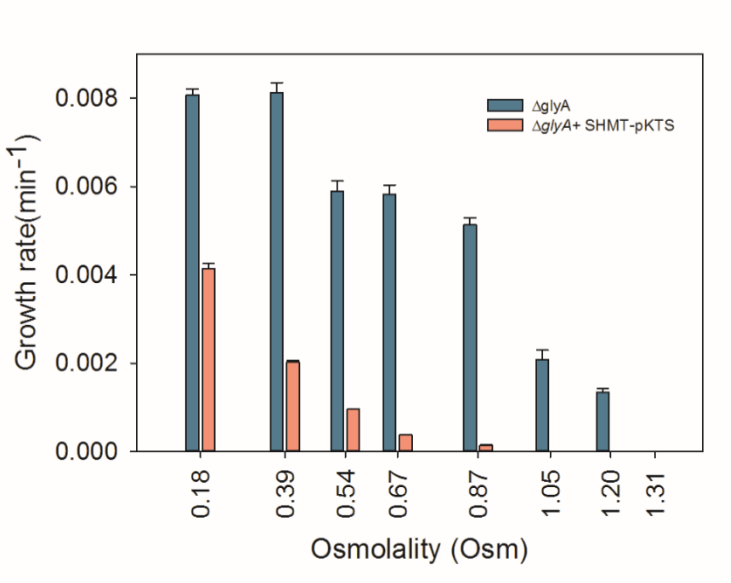



**Figure S9.** The growth of deletant and complemented cells of the Δ*glyA* strain using NaCl as the osmotic stressor. Panel A shows the growth of cells in BV media supplemented with glycine and serine as well as kanamycin. The cells show growth up to 1.15 Osm. Panel B shows the growth in BV minimal media with kanamycin, ampicillin and 75ng/ml tetracycline. The SHMT-pKTS clone allows cells to grow confluently until 0.48 Osm. Isolated overgrowing colonies appear until 0.70 Osm. The cartoons on the right provide a template for the position of the different strains.

**A.**




**B.**

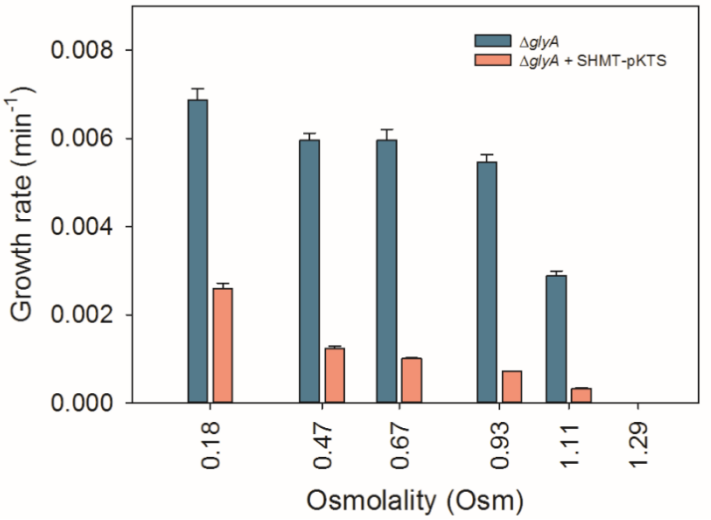



**Figure S10.** The activity of *ΔglyA E. coli* carrying the serine hydroxymethyl transferase gene cloned in the pKTS plasmid can be titrated with increasing *in vivo* osmotic stress. Panel A (left) plots the growth rate ($min^{-1}$) of the Δ*glyA* strain (green bar) vs. osmolality in sorbitol containing media while Panel B (right) shows the growth pattern in NaCl containing media. The deletion strain was grown in BV media supplemented with glycine and serine (green bar) whereas the Δ*glyA* + SHMT-pKTS complemented cells (red bar) were grown in minimal media with 50ngml tetracycline.

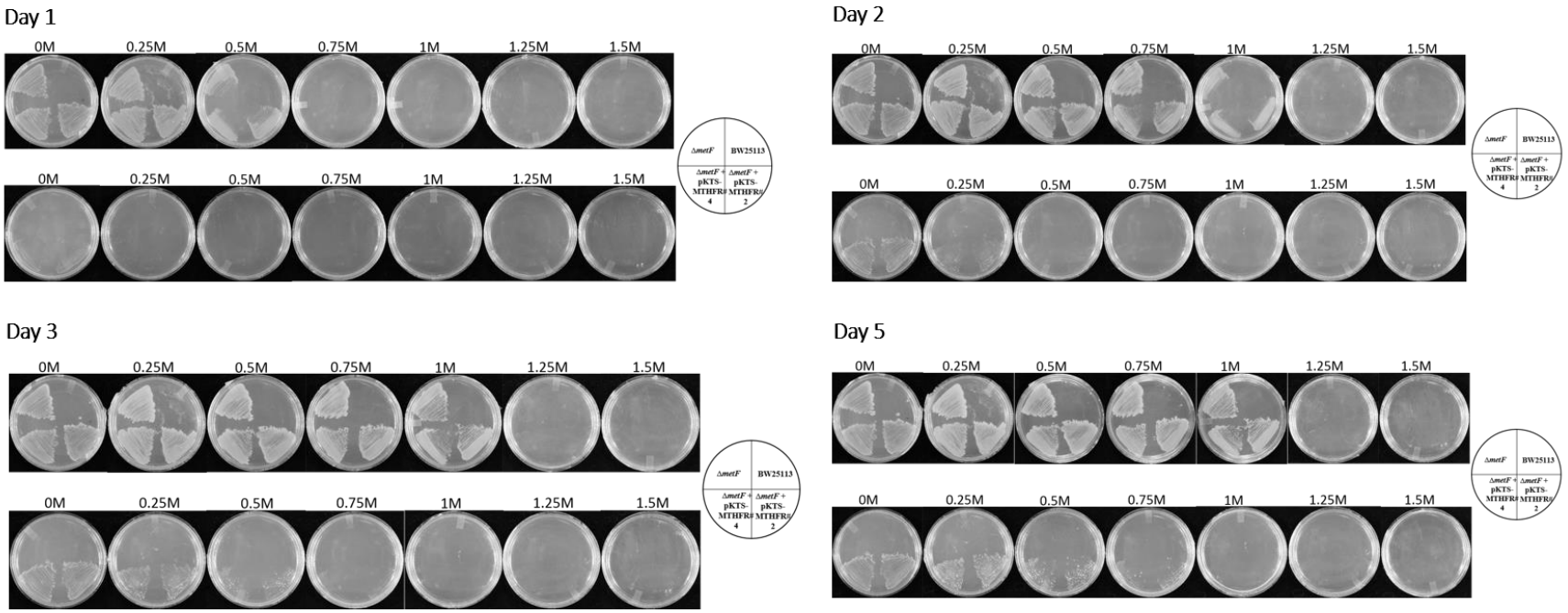



**Figure S11.** Time course of the growth of *ΔmetF* cells that are complemented by the MTHFR –pKTS plasmid. Cells were grown on BV media supplemented with methionine on the top panel and BV minimal media with 100ng/ml tetracycline on the bottom panel. The sorbitol concentrations used are listed on top of each plate. The plates were monitored every day for a span of five days. Cell growth on the minimal plates becomes confluent by 2-3 days and stays the same for 5 days. Isolated overgrowing colonies appeared around day 2-3.

**Table S3: List of s*uppressors appearing at or near the SsrA tag***

| | |
|---|---|
| OverGrowerR67DHFR#7 | Mutation in Xho1 site. SsrA tag not made |
| OverGrowerR67DHFR#9 | Mutation in Xho1 site. SsrA tag not made. Different site than OG#7. |
| OverGrowerR67DHFR#11 | Stop codon introduced in Xho1 Site. |
| OverGrowerR67DHFR#15 | Stop codon introduced in Xho1 Site. |
| OverGrowerR67DHFR#19 | Stop codon introduced in Xho1 Site. |
| OverGrowerR67DHFR#20 | Stop codon introduced in Xho1 Site. |
| | |
| OverGrowerMTHFR#1 | Stop codon in *metF* gene sequence, three amino acids before termination. No SsrA tag made. |
| OverGrowerMTHFR#2 | Frameshift mutation in Xho1 restriction site. SsrA tag not made. |
| OverGrowerMTHFR#4 | Base deletion in the *metF* gene sequence, 5 amino acids before termination. No SsrA tag made. |
| OverGrowerMTHFR#5 | Bases introduced in SsrA tag leading to stop codon TGA where the 4$^{th}$ amino acid of SsrA tag would be. No tag made. |
| OverGrowerMTHFR#6 | Stop codon in *metF* gene sequence in the last amino acid before termination. Thus, no SsrA tag made. |
| OverGrowerMTHFR#7 | Stop codon TGA introduced in Xho1 restriction site. No SsrA tag made. |
| OverGrowerMTHFR#9 | Bases introduced in SsrA tag leading to stop codon TGA in the 4$^{th}$ amino acid of SsrA tag. No tag made. |
| | |
| OverGrowerSHMT#2 | Frameshift mutation in SsrAtag. The tag is not made. |
| OverGrowerSHMT#4 | Stop codon introduced in SsrA tag. |
| OverGrowerSHMT#10 | Stop codon introduced in SsrA tag. |
| OverGrowerSHMT#11 | Stop codon introduced in SsrA tag. |

**Table S4.** Predicted $\mu_{23}$/RT values for various ligands associated with the enzymes studied. Substrates are listed in black; products are listed in blue; inhibitors are listed in red and pathway end products are listed in green.

| Enzyme | Ligand | Betaine $\mu_{23}$/RT ($m^{-1}$)[a] | Trehalose $\mu_{23}$/RT ($m^{-1}$) [b] | Glutamate $\mu_{23}$/RT ($m^{-1}$) [c] | LogP [d] |
|---|---|---|---|---|---|
| DHFR | NADPH | 0.983 | -0.408 | 0.781 | -7.183 |
| | DHF | -0.049 | -0.110 | 0.650 | -3.409 |
| | $NADP^+$ | 1.051 | -0.768 | 0.815 | -7.417 |
| | THF | -0.112 | -0.122 | 0.547 | -3.892 |
| | Trimethoprim | -0.744 | 0.468 | 0.568 | 1.258 |
| Serine-hydroxymethyl transferase | Serine | 0.160 | 0.018 | 0.080 | -3.661 |
| | Glycine | 0.156 | -0.038 | 0.020 | -3.022 |
| | THF | -0.112 | -0.122 | 0.547 | -3.892 |
| | 5,10-methyleneTHF | -0.053 | -0.105 | 0.712 | -3.814 |
| | Pyridoxal 5'-phosphate | 0.820 | -0.676 | 0.422 | -1.550 |
| Chorismate mutase | Chorismate | 0.430 | 0.140 | 0.508 | -2.758 |
| | Prephenate | 0.563 | -0.006 | 0.451 | -3.081 |
| | Tyrosine | -0.200 | 0.007 | 0.136 | -1.705 |
| | Phenylalanine | -0.340 | 0.029 | 0.307 | -1.411 |
| Methylene tetrahydrofolate reductase | NADH | 0.160 | 0.277 | 0.766 | -4.967 |
| | 5,10-methyleneTHF | -0.053 | -0.105 | 0.712 | -3.814 |
| | FAD | 0.999 | -0.651 | 1.159 | -5.090 |
| | 5-methyl-THF | -0.089 | -0.145 | 0.694 | -3.550 |
| | $NAD^+$ | -0.169 | 0.112 | 0.793 | -5.200 |

[a] Values calculated using equation 4 from reference (4).
[b] Values calculated from reference (5).
[c] Values calculated from reference (6).
[d] LogP values were calculated using MOE version 2015.1001.

**Cloning into the pKTS plasmid** As a first step in our cloning process, we obtained the *E. coli* methylene tetrahydrofolate reductase gene carried in the pCAS30 plasmid (7) from Elizabeth Trimmer (Grinnell College). The pBSGlyA plasmid obtained from Roberto Contestable (Sapienza Università di Roma) encoded serine hydroxymethyl-transferase from *E. coli* (8-10). The Quad4 gene (11) was synthesized by Genscript and supplied as a clone in pUC57.

To clone these various folate pathway genes into the pKTS plasmid, NdeI and XhoI restriction enzyme sites were required. The NdeI site occurs at the 5' end of the gene (CATATG with the ATG being the start methionine codon) and the XhoI site marks the 3' end of the gene and the beginning of the sequence encoding the SsrA degradation tag in pKTS. PCR was used to incorporate these restriction sites into the pBSGlyA plasmid. A XhoI site already existed at the end of the *metF* gene in the pCAS30 plasmid. Primers are given in Table 4. The presence of the restriction sites was verified by DNA sequencing at the Molecular Biology Resource facility (University of Tennessee, Knoxville).

The NdeI-XhoI fragments were then ligated into the pKTS vector and electroporated into the appropriate deletant strains of *E. coli*. An internal XhoI site was present in the *glyA* gene, thus a partial digest was used for cloning into the pKTS plasmid. To identify the correct clones, the selection used the ability of the clone to rescue the host strain from auxotrophy. An alternate selection used YT agar with a selective antibiotic. The first selection is based on the ability of the clones to rescue the host to prototrophy and the second selection allows for antibiotic resistance selection either associated with the pKTS vector or the inserted gene. The correct clones were verified by DNA sequencing. Additional details are provided in reference (12).

**Method to Measure Colony Forming Units:** The cells were grown overnight in Luria-Bertani broth and centrifuged the next day. The resulting pellet was washed with 1X Bonner-Vogel (BV) salts (13). These steps were repeated and the cell pellet resuspended in 1X BV. The knockout and rescued cells were inoculated into 3ml of BV supplemented and BV minimal media at an OD of 0.2 at 600nm. Different concentrations of sorbitol were used. 20µl of cells were taken from each tube for dilutions at 0, 24 and 40 hours. Serial dilutions were performed by taking 20µl of cells and adding the aliquot to 180µl of medium. 10µl of each of these dilutions were spotted onto LB plates containing the appropriate antibiotics and plates were incubated overnight at 37°C. CFU were counted the next day and colony numbers of deletion and complemented strains were compared at each sorbitol concentration to check for stasis or cell death.

**References**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