Supplementary Information (SI) for Analytical Methods.


# Supplementary Information

## In-cell NMR Reveals Metabolic Adaptations in Central Carbon Pathways Driving Antibiotic Tolerance in *Salmonella* Typhimurium

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## Supplementary Figures:

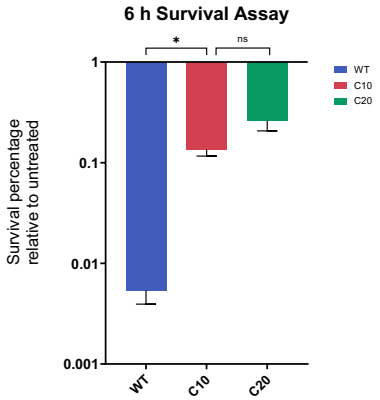


**Figure S1. Six-hour survival assay of *S.* Typhimurium after 20 cycles of ampicillin treatment.** After 10 cycles of ampicillin treatment, we observed a significant increase in tolerance in the evolved C10 strain compared to the wild type (WT). To assess whether tolerance could be further enhanced, the C10 strain was subjected to an additional 10 cycles of ampicillin treatment, resulting in the C20 strain. Survival of the C20 strain was evaluated using a 6-hour survival assay. Overnight cultures of WT, C10, and C20 were centrifuged, resuspended in pure GAM medium or GAM supplemented with 500 µg/mL ampicillin, and incubated for 6 h at 37 °C with shaking (200 rpm). Cultures were then plated to determine colony-forming units (CFU), and survival was calculated relative to the untreated control. While the C10 strain displayed significantly increased survival compared to WT ($p < 0.05$), no further significant increase in survival was observed in the C20 strain. This indicates the enhancement of tolerance plateaus after 10 cycles of treatment.

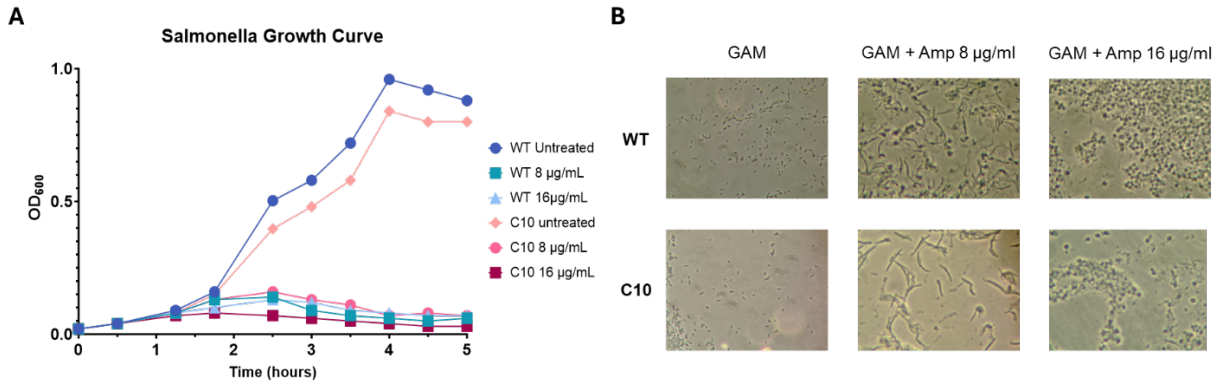


**Figure S2. Growth curve and morphology of *Salmonella* WT and C10 strains in the presence or absence of ampicillin.** (A) The growth of *S.* Typhimurium wild-type (WT) and C10 strains was monitored by measuring $OD_{600}$ at 30 min intervals. Both strains were grown in GAM medium with and without ampicillin (8 µg/mL or 16 µg/mL), starting from an initial $OD_{600}$ of 0.02. Growth was significantly inhibited by both concentrations of ampicillin, with cell density reaching its peak at around 2.5 h. There was no significant difference in growth inhibition between the WT and C10 strains under ampicillin treatment. (B) Brightfield microscopy images of WT and C10 cells after 2.5 h of growth, showing untreated and ampicillin-treated conditions. Untreated WT and C10 cells exhibited the typical rod-shaped morphology. Under treatment with 8 µg/mL ampicillin, both strains showed elongation of cells, indicative of reduced cell division under antibiotic pressure. At 16 µg/mL ampicillin, significant cell lysis was observed in both strains, indicating cell death at this time point. No substantial differences in morphology were observed between the WT and C10 strains under any condition.

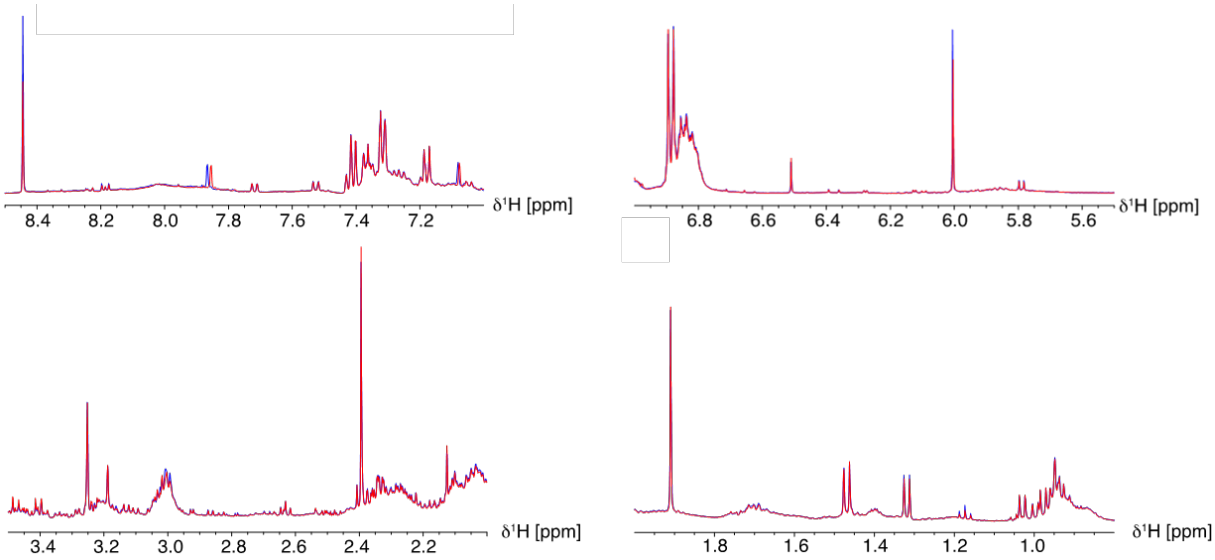


**Figure S3. $^{1}$H NMR spectra of *S.* Typhimurium WT and C10 after 2.5 h of growth in GAM medium.** Representative $^{1}$H NMR spectra of *S.* Typhimurium wild-type (WT, blue) and C10 (red) strains after 2.5 h of growth in GAM medium. The overlayed spectra demonstrate the metabolic similarities between the WT and C10 strains, indicating overall comparable levels of metabolite abundance under these growth conditions.

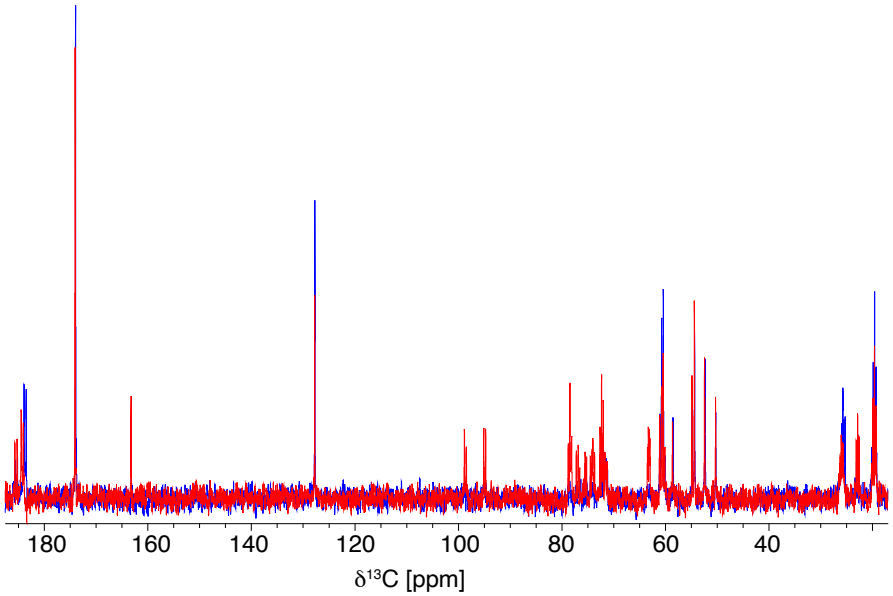


**Figure S4. In-cell $^{13}$C NMR spectra of exponentially growing *S.* Typhimurium WT and C10 strains following [U-$^{13}$C] glucose injection.** $^{13}$C NMR spectra of wild-type (WT, blue) and tolerance-evolved (C10, red) *S.* Typhimurium strains, harvested at mid-log phase and analyzed by in-cell NMR. Spectra were recorded at approximately 1 min intervals in a pseudo-2D experiment, with the data shown here capturing metabolite levels 15 minutes after injection of the $^{13}$C-labeled substrate.

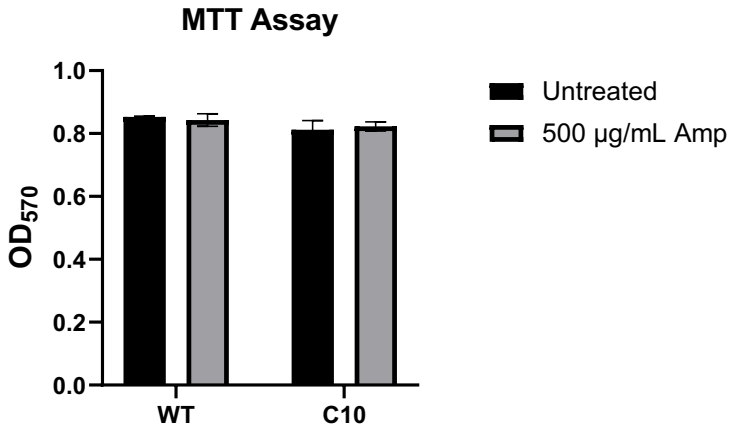


**Figure S5. Impact of Ampicillin on Cell Viability in *S. Typhimurium* Strains.** The viability of *S. Typhimurium* wild-type (WT) and C10 was evaluated following a 10 min exposure to 500 µg/mL ampicillin using an MTT assay. In this assay, mitochondrial dehydrogenases in viable cells reduce MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) to form a colored product, serving as an indicator of cell viability measured by optical density. Viability was compared between treated and untreated cells, and no significant reduction in viability was observed in either strain within the first 10 minutes of ampicillin exposure, suggesting that this initial exposure level did not impact cell viability under the tested conditions.

**MTT Assay Protocol.** Overnight cultures of the wild type (WT) and C10 were diluted to an optical density $OD_{600}$ of 0.02 in 45 mL of GAM medium and grown for 2.5 h at 37 °C with shaking at 120 rpm. After incubation, a volume corresponding in number of cells to 1 mL with an $OD_{600}$ of 15 was harvested from each culture into 50 mL conical tubes. The cultures were centrifuged, and the cell pellets were washed in 5 mL of HEPES buffer before being resuspended in 600 µL of HEPES buffer. Of the resuspended cells, 300 µL were transferred into two Eppendorf tubes. To one of the tubes, 500 µg/mL of ampicillin was added, and a timer was set for 10 min. After incubation, the tubes were centrifuged, and the supernatant was discarded. An MTT solution was prepared in TSB to achieve a final working concentration of 0.05 mg/mL (0.3 mg of MTT dissolved in 6 mL of TSB). The cell pellet was resuspended in 500 µL of the MTT solution and incubated at 37 °C with shaking at 200 rpm. After 30 min, samples were checked for color development, indicating viable cells. The samples were centrifuged again, and the supernatant was discarded. The pellets were resuspended in 500 µL of DMSO, and absorbance was measured at 570 nm.