**Supplementary figure legends**

**Figure S1**. Drug dose-response curves for wild type (grey) and Δlon::Kan (black) E. coli against ampicillin, rifampicin, nalidixic acid and chloramphenicol. Peak optical density after 15-18 hours of growth at each trimethoprim concentration was normalized to growth in drug-free media (normalized growth). Mean ± SD of 3 independent experiments are plotted.

**Figure S2.** List of single base substitutions and their respective amino acid changes at 3 hotspots of trimethoprim resistance evolution in DHFR. Mutants that were not generated or not used in this study are greyed out.

**Figure S3.** The 3 hotspots chosen for mutational analyses are shown in the structure of DHFR (PDB:7DFR). The DHFR polypeptide is shown in cartoon representation (grey) while Pro21, Trp30 and Ile94 are shown as sticks and coloured by element (C: green O: red N: blue). Folate and NADP bound to the active site are shown as lines and coloured by element (C: magenta O: orange P: red N: blue). Pro21 is found in the Met20 catalytic loop (red). Trp30 in found on the buried face of an α-helix (blue). Ile94 is an active site residue.

**Figure S4.** The size of the clipped fragment of Trp30Ser DHFR in Lon-expressing bacteria was calculated based on electrophoretic mobility. A standard was generated using known molecular weights and their Rf values as shown. The Rf values for wild type and Trp30Ser DHFR were calculated and their molecular weights were inferred based on the standard.

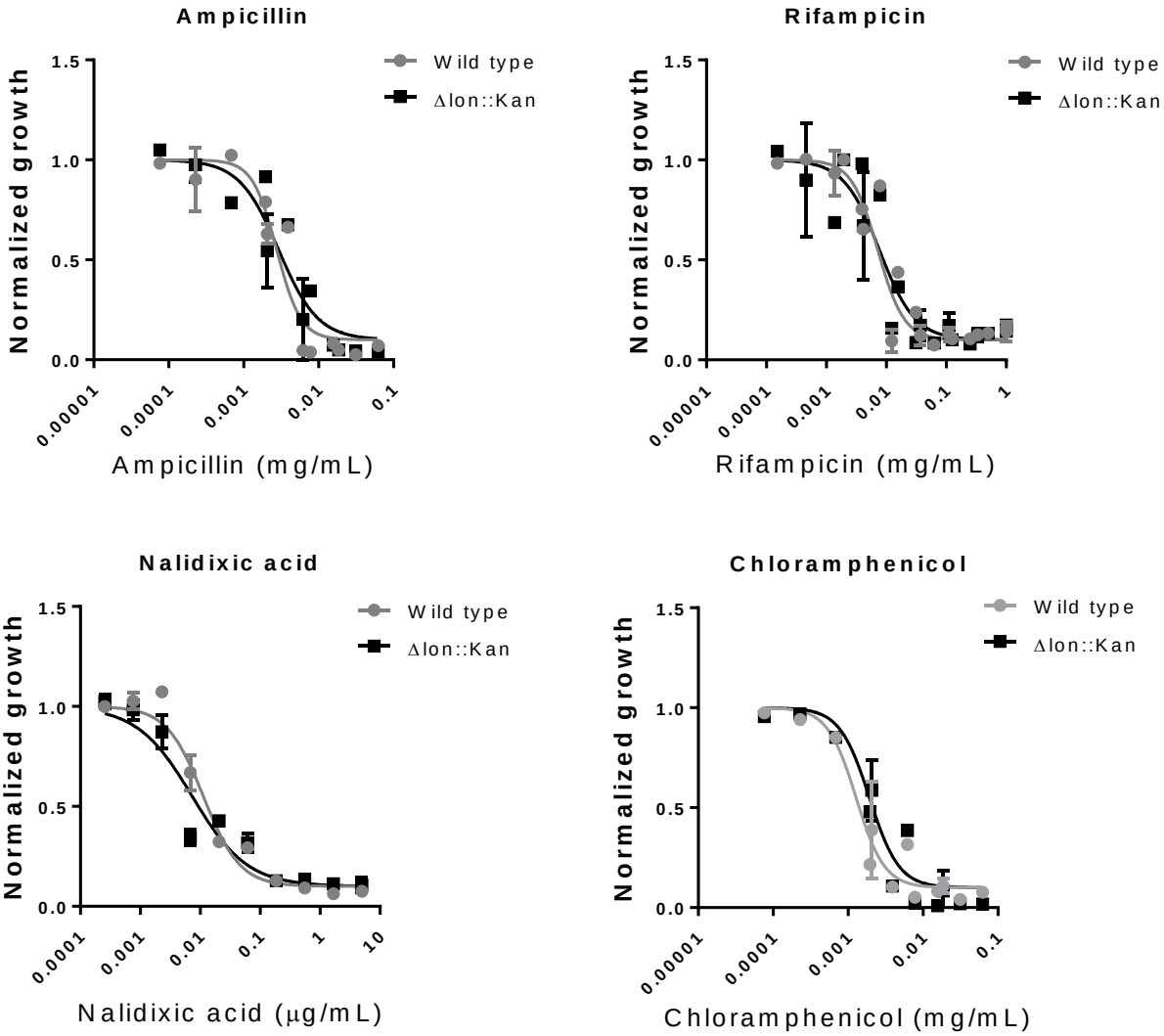


**Figure S1. Matange N.**

Wild type ⇨

| Pro21 | | Trp30 | | Ile94 | |
|---|---|---|---|---|---|
| CCG | Pro | TGG | Trp | ATT | Ile |
| ACG | Thr | AGG | Arg | GTT | Val |
| GCG | Ala | GGG | Gly | TTT | Phe |
| TCG | Ser | CGG | Arg | CTT | Leu |
| CAG | Gln | TAG | Stop | AGT | Ser |
| CGG | Arg | TTG | Leu | AAT | Asn |
| CTG | Leu | TCG | Ser | ACT | Thr |
| CCA | Pro | TGA | Stop | ATG | Met |
| CCC | Pro | TGT | Cys | ATA | Ile |
| CCG | Pro | TGC | Cys | ATC | Ile |

**Figure S2. Matange N.**

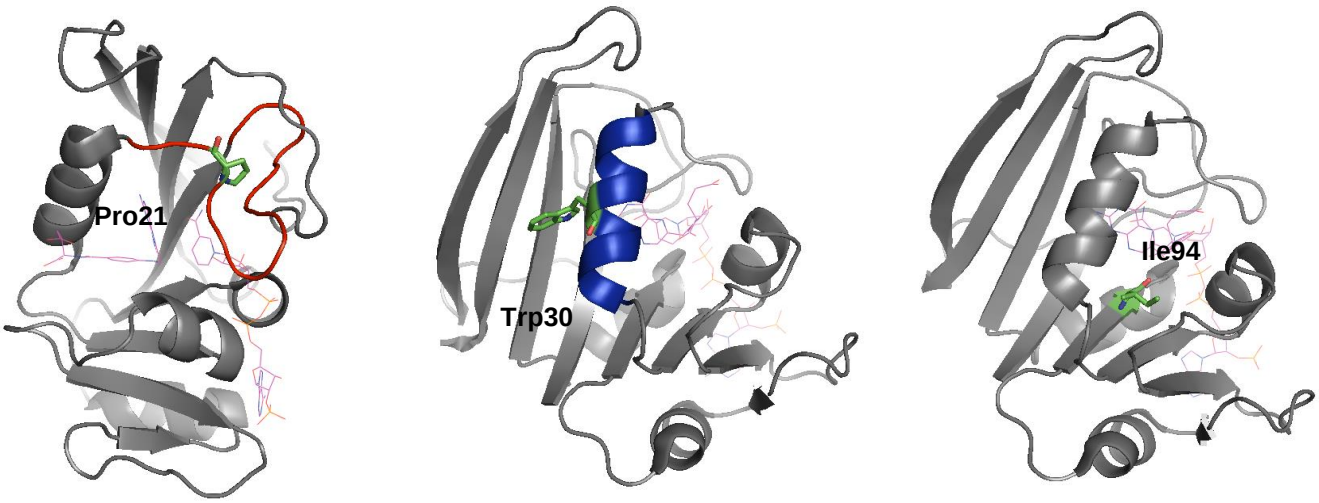


**Figure S3. Matange N.**

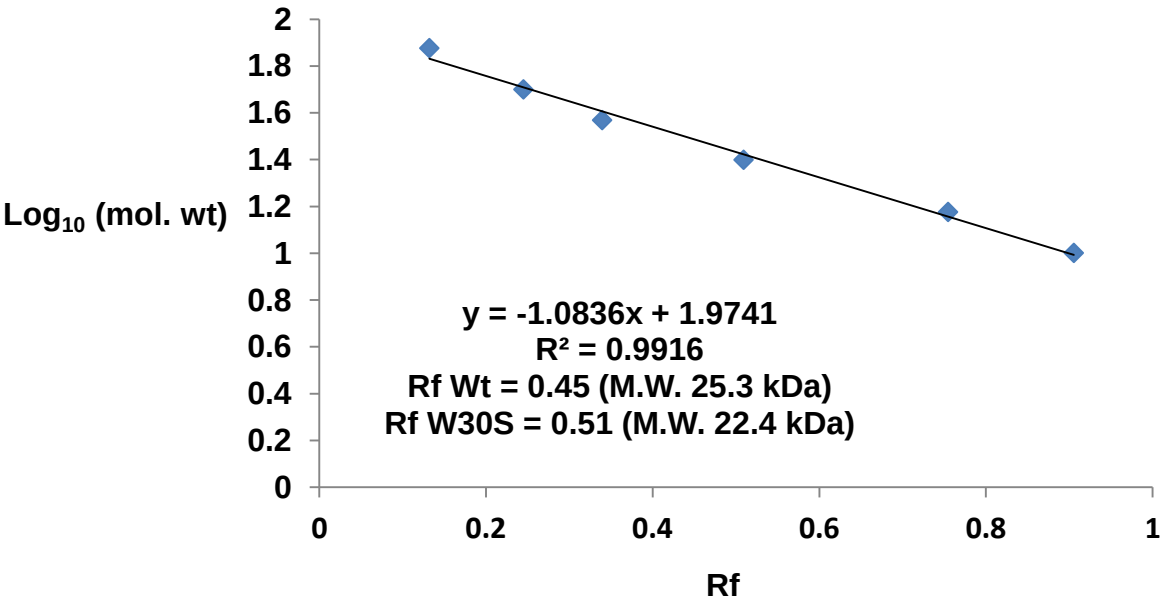


**Figure S4. Matange N.**