The chemotherapeutic drug methotrexate selects for antibiotic resistance

Jónína S. Gudmundsdóttir[1], Elizabeth G. A. Fredheim[1], Catharina I. M. Koumans[2], Joachim Hegstad[1,5], Po-Cheng Tang[3], Dan I. Andersson[3], Ørjan Samuelsen[1,4] and Pål J. Johnsen[1*]

[1]Department of Pharmacy, Faculty of Health Sciences, UiT The Arctic University of Norway, Tromsø, Norway.

[2]Institute of Biology, Leiden University, Leiden, The Netherlands.

[3]Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden.

[4]Norwegian National Advisory Unit on Detection of Antimicrobial Resistance, [5]Department of Microbiology and Infection Control, University Hospital of North Norway, Tromsø, Norway.

*Correspondence to: paal.johnsen@uit.no

Abstract:

Many cytotoxic drugs used in cancer chemotherapy have antibacterial properties, but how bacterial populations are affected by these selective pressures is unknown. Here we test the hypothesis that the widely used cytotoxic drug methotrexate affects the evolution and selection of antibiotic resistance. We show that methotrexate can select for trimethoprim resistance determinants located on the chromosome or a plasmid. Additionally, methotrexate can co-select for virtually any antibiotic resistance determinant when present together with trimethoprim resistance on a multidrug-resistance clinical plasmid. These selective effects occur at concentrations 40- to >320-fold below the methotrexate minimal inhibitory concentration for *Escherichia coli,* suggesting a selective role of methotrexate chemotherapy for antibiotic resistance in patients that strongly depend on effective antibiotic treatment.

One Sentence Summary:

Methotrexate, a drug used in the treatments of cancers and inflammatory diseases, selects and co-selects antibiotic resistance determinants.

Main Text:

Antibiotic resistance is a major risk factor for patients with impaired immunity, such as cancer patients, and often a patient's survival depends on antibiotic treatment to reduce the risk for hospital-acquired infections during chemotherapy (*1, 2*). Paradoxically, evidence suggest that cytotoxic drugs used in cancer chemotherapy may promote antibiotic resistance through cross-resistance (*3*) and *de novo* evolution through SOS induced mutagenesis (*4-8*). There is an urgent need to understand these potential collateral effects of cancer chemotherapy to ensure effective antibiotic treatment for a large group of immunocompromised patients. Moreover, cytotoxic drugs may constitute a previously unrecognized target for intervention to limit the selection and

spread of antibiotic resistance. Here we explored how selective and co-selective pressures
45 exerted by the cytotoxic drug methotrexate (MTX) affect the enrichment and evolution of antibiotic resistance determinants.

MTX is a cytotoxic drug structurally similar to the antibiotic trimethoprim (TMP). Both drugs act through inhibiting the dihydrofolate reductase enzyme in bacteria and eukaryotic cells (*9*). MTX is widely used in treatments including but not limited to; cancer of the breast, skin,
50 head, neck, and lung as well as many inflammatory diseases, such as rheumatoid arthritis (*10*), making it one of the most commonly prescribed drugs in the USA (*11*).

We asked if exposure to MTX selects for pre-existing TMP resistance (TMP^R) determinants in clinical and laboratory strains of *Escherichia coli* (Table S1), the most frequently isolated bacterial species from urinary tract (UTIs) and blood stream infections (*12*).
55 TMP is among the most frequently used antibiotics in the treatment of UTIs. Initial experiments revealed variable, TMP^R dependent, but consistently high MTX minimal inhibitory concentrations (MICs) (Table S2). This is consistent with previous reports indicating that *E. coli* displays reduced susceptibility towards MTX due to AcrAB-TolC mediated efflux (*3, 13*).

Antibiotic resistance selection and co-selection have traditionally been assumed to
60 occur between the MICs of susceptible and resistant isolates (known as the selective window) within a bacterial population (*14*). However, several reports unequivocally show that antibiotic resistance selection and co-selection can occur at concentrations several hundred-fold below the MICs of the susceptible isolate (known as sub-MICs) (*14-16*). To test how sub-MICs of MTX affect bacterial fitness, we measured exponential growth rates for two pairs of clinical
65 isogenic TMP^R and susceptible (TMP^S) *E. coli* across a wide MTX concentration span. One pair with TMP^R located on the chromosome (two mutations associated with *folA*) and one pair with TMP^R (*dfrA*) located on the multidrug-resistance (MDR) plasmid pG06-VIM-1 (*17, 18*) (see Materials and Methods for detailed description of the strains). TMP^S strains displayed

sharply declining growth rates between 1 and 2 mg/mL of MTX, whereas the TMP^R strains
70 remained unaffected (Figure S1). These results suggest a selective benefit during MTX exposure for TMP^R strains at concentrations below the observed MIC of MTX for the TMP susceptible clinical isolates.

The minimum selective concentration (MSC) describes pharmacodynamically the lowest concentration where selection for resistance occurs (*14*). To determine the MSC for
75 MTX, we constructed a pair of *E. coli* MG1655 strains with yellow (*sYFP2*) and blue (*mTagBFP2*) fluorescent proteins to enable accurate separation between the two in mixed populations. In these backgrounds, we introduced chromosomal TMP^R mutations (*folA*) using genome engineering (*19*) and moved the pG06-VIM-1 plasmid into the fluorescence-tagged strains. The isogenic TMP^R and TMP^S strain pairs were competed head-to-head by serial
80 passage for 30 generations and the ratio of TMP^R:TMP^S was determined over time using flow cytometry. From this data the MSC was estimated (Tables S3-S5) (*14*). *E. coli* with chromosomal *folA* mutations had an MSC of 200 μg/mL (1/40 MIC of MTX) whereas those harboring the plasmid were close to the detection limit of the assay (*14*) and we conservatively estimated the MSC to be <25 μg/mL (less than 1/320 MIC of MTX) (Figure 1). The phenotypic
85 and genotypic stability of the MDR pG06-VIM-1 plasmid (SI) (*18*) further demonstrate that MTX-mediated selection enables co-selection of associated resistance determinants.

Exploring MTX-selective dynamics further, we asked if TMP^R determinants could invade the population at lower initial densities to exclude potential bias from the 1:1 ratio in the competition experiments. We started competition experiments from frequencies as low as 10^{-4}
90 of the TMP^R strains, at concentrations slightly above the estimated MSC of MTX (400 μg/mL for *folA* mediated resistance and 75 μg/mL for pG06-VIM-1 mediated resistance) and followed the change in ratios over 30 generations of growth (Figure 2). Both chromosomal and plasmid mediated TMP^R determinants were able to invade, even when initially rare in their respective

populations, strongly suggesting that the MTX-selective effects are independent on initial
95 frequencies of resistant and susceptible strains during competition experiments.

We further examined whether exposure to MTX could lead to *de novo* TMP^R evolution. We selected spontaneous mutants from overnight cultures with and without exposure to MTX, plated on selective agar at high MTX concentrations and checked for TMP cross-resistance (Figure S2, Table S6). *E. coli* K56-2 isolated at 16 and 32 mg/mL MTX (MP18-17 to MP18-
100 28) displayed increased MICs of TMP close to or above the clinical breakpoint (*20*), clearly demonstrating selection for TMP^R by MTX at clinically relevant drug levels. DNA sequencing of the resistant isolates revealed two different mutations in the *folA* promoter, previously reported to result in TMP^R (*21*), as well as a single mutation in the *marR* gene (Table S6-S11).

Finally, we asked if exposure to sub-MICs of MTX close to the estimated MSC's would
105 select for *de novo* TMP mutations in a susceptible *E. coli* population. Starting from 1,000 cells to minimize the probability of pre-existing mutants, we grew 10 independent lineages of the *E. coli* K56-2 strain at 400 μg/mL MTX for 500 generations. The frequency of TMP^R was determined every 50 generations. TMP^R ascended in frequency in 2/10 lineages at different rates and time-points during the first 250 generations before they were outcompeted by a
110 different set of mutants with reduced susceptibility to MTX and no cross-resistance to TMP (Figure 3, Table S12). These experiments show that MTX exposure can select for *de novo* TMP^R both at sub-MIC and at higher concentrations.

To assess pharmacokinetic relevance, we used the limited data available on intestinal MTX concentrations. We estimated that MTX dosing required to achieve intestinal
115 concentrations at and above MSCs (range 0.15-3.75g/m^2) reflects expected concentration ranges obtained during high dose MTX treatment (0.5-12 g/m^2) (see Supplementary Text for details) (*3, 22*).

Altogether, we show that exposure to the cytotoxic drug MTX affects selection and evolution of TMP^R determinants at clinically relevant concentrations. Notably, MTX can mediate selection of any antibiotic resistance determinant when TMP^R is co-localized on a mobile genetic element (often the case in clinical *E. coli* isolates). This potentially important side-effect of MTX treatment has been previously unrecognized, as studies on the effects of non-antibacterial drugs, including MTX, have either focused on bacterial growth inhibition or used drug concentrations around the MIC (*3, 23-25*), with a few exceptions (*15, 26*). To acquire a deeper understanding of the evolutionary potential of novel, non-antibiotic drivers of antibiotic resistance the approaches presented here are essential. Combined with an improved understanding of the intestinal pharmacokinetics of MTX and other cytotoxic drugs and their interactions with the human microbiome, such knowledge could allow identification of antibiotic + non-antibiotic drug combinations that should be avoided to preempt resistance evolution.

References and Notes:

1. A. Teillant, S. Gandra, D. Barter, D. J. Morgan, R. Laxminarayan, Potential burden of antibiotic resistance on surgery and cancer chemotherapy antibiotic prophylaxis in the USA: a literature review and modelling study. *Lancet Infectious Diseases* **15**, 1429-1437 (2015).
2. P. A. Danai, M. Moss, D. M. Mannino, G. S. Martin, The epidemiology of sepsis in patients with malignancy. *Chest* **129**, 1432-1440 (2006).
3. L. Maier *et al.*, Extensive impact of non-antibiotic drugs on human gut bacteria. *Nature* **555**, 623-628 (2018).
4. L. E. Papanicolas, D. L. Gordon, S. L. Wesselingh, G. B. Rogers, Not just antibiotics: is cancer chemotherapy driving antimicrobial resistance? *Trends in Microbiology* **26**, 393-400 (2018).
5. A. Meunier *et al.*, Enhanced emergence of antibiotic-resistant pathogenic bacteria after *in vitro* induction with cancer chemotherapy drugs. *Journal of Antimicrobial Chemotherapy* **74**, 1572-1577 (2019).
6. C. A. Hobson *et al.*, Impact of anticancer chemotherapy on the extension of β-lactamase spectrum: an example with KPC-type carbapenemase activity towards ceftazidime-avibactam. *Scientific Reports* **10**, 589 (2020).
7. S.-i. Nakamura, Y. Oda, T. Shimada, I. Oki, K. Sugimoto, SOS-inducing activity of chemical carcinogens and mutagens in *Salmonella typhimurium* TA1535/pSK1002: examination with 151 chemicals. *Mutation Research Letters* **192**, 239-246 (1987).

8. G. E. Foley *et al.*, A comparative study of the use of microorganisms in the screening of potential antitumor agents. *Annals of the New York Academy of Sciences* **76**, 413-438; discussion 438-441 (1958).
9. R. V. Mauldin, M. J. Carroll, A. L. Lee, Dynamic dysfunction in dihydrofolate reductase results from antifolate drug binding: modulation of dynamics within a structural state. *Structure* **17**, 386-394 (2009).
10. J. Jolivet, K. H. Cowan, G. A. Curt, N. J. Clendeninn, B. A. Chabner, The pharmacology and clinical use of methotrexate. *New England Journal of Medicine* **309**, 1094-1104 (1983).
11. S. P. Kane, "ClinCalc DrugStats Database, Version 20.1," (ClinCalc LLC, 2020; https://clincalc.com/DrugStats).
12. M. J. Struelens, O. Denis, H. Rodriguez-Villalobos, Microbiology of nosocomial infections: progress and challenges. *Microbes and Infection* **6**, 1043-1048 (2004).
13. S. J. Kopytek, J. C. Dyer, G. S. Knapp, J. C. Hu, Resistance to methotrexate due to AcrAB-dependent export from *Escherichia coli*. *Antimicrobial Agents and Chemotherapy* **44**, 3210-3212 (2000).
14. E. Gullberg *et al.*, Selection of resistant bacteria at very low antibiotic concentrations. *PLOS Pathogens* **7**, e1002158 (2011).
15. E. Gullberg, L. M. Albrecht, C. Karlsson, L. Sandegren, D. I. Andersson, Selection of a multidrug resistance plasmid by sublethal levels of antibiotics and heavy metals. *mBio* **5**, e01918-01914 (2014).
16. S. Westhoff *et al.*, The evolution of no-cost resistance at sub-MIC concentrations of streptomycin in *Streptomyces coelicolor*. *The ISME Journal* **11**, 1168-1178 (2017).
17. N. L. Podnecky *et al.*, Conserved collateral antibiotic susceptibility networks in diverse clinical strains of *Escherichia coli*. *Nature Communications* **9**, 3673 (2018).
18. M. C. Di Luca *et al.*, Low biological cost of carbapenemase-encoding plasmids following transfer from *Klebsiella pneumoniae* to *Escherichia coli*. *Journal of Antimicrobial Chemotherapy* **72**, 85-89 (2016).
19. Á. Nyerges *et al.*, A highly precise and portable genome engineering method allows comparison of mutational effects across bacterial species. *Proceedings of the National Academy of Sciences* **113**, 2502-2507 (2016).
20. The European Committee on Antimicrobial Susceptibility Testing, "Breakpoint tables for interpretation of MICs and zone diameters. Version 10.0," (EUCAST, 2020; http://www.eucast.org).
21. G. M. Eliopoulos, P. Huovinen, Resistance to trimethoprim-sulfamethoxazole. *Clinical Infectious Diseases* **32**, 1608-1614 (2001).
22. E. S. Henderson, R. H. Adamson, V. T. Oliverio, The metabolic fate of tritiated methotrexate. II. Absorption and excretion in man. *Cancer Research* **25**, 1018-1024 (1965).
23. H. Kruszewska, T. Zareba, S. Tyski, Antimicrobial activity of selected non-antibiotics - Activity of methotrexate against *Staphylococcus aureus* strains. *Acta Poloniae Pharmaceutica* **57 Suppl**, 117-119 (2000).
24. M. R. Moody *et al.*, Effect of two cancer chemotherapeutic agents on the antibacterial activity of three antimicrobial agents. *Antimicrobial Agents and Chemotherapy* **14**, 737-742 (1978).
25. A. Nyhlén, B. Ljungberg, I. Nilsson-Ehle, I. Odenholt, Bactericidal effect of combinations of antibiotic and antineoplastic agents against *Staphylococcus aureus* and *Escherichia coli*. *Chemotherapy* **48**, 71-77 (2002).

26. Y. Wang *et al.*, Antiepileptic drug carbamazepine promotes horizontal transfer of plasmid-borne multi-antibiotic resistance genes within and across bacterial genera. *The ISME Journal* **13**, 509-522 (2019).
27. G. Kahlmeter, The ECO.SENS Project: a prospective, multinational, multicentre epidemiological survey of the prevalence and antimicrobial susceptibility of urinary tract pathogens--interim report. *Journal of Antimicrobial Chemotherapy* **46 Suppl 1**, 15-22; discussion 63-15 (2000).
28. H. Ikeda, J.-i. Tomizawa, Transducing fragments in generalized transduction by phage P1: I. Molecular origin of the fragments. *Journal of Molecular Biology* **14**, 85-109 (1965).
29. G. Bertani, Studies on lysogenesis. I. The mode of phage liberation by lysogenic *Escherichia coli*. *Journal of Bacteriology* **62**, 293-300 (1951).
30. K. A. Datsenko, B. L. Wanner, One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences* **97**, 6640 (2000).
31. D. Yu *et al.*, An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proceedings of the National Academy of Sciences of the United States of America* **97**, 5978-5983 (2000).
32. S. K. Sharan, L. C. Thomason, S. G. Kuznetsov, D. L. Court, Recombineering: a homologous recombination-based method of genetic engineering. *Nature Protocols* **4**, 206-223 (2009).
33. J. Näsvall, A. Knöppel, D. I. Andersson, Duplication-Insertion Recombineering: a fast and scar-free method for efficient transfer of multiple mutations in bacteria. *Nucleic Acids Research* **45**, e33 (2017).
34. Q. Tu *et al.*, Room temperature electrocompetent bacterial cells improve DNA transformation and recombineering efficiency. *Scientific Reports* **6**, 24648 (2016).
35. L. M. Guzman, D. Belin, M. J. Carson, J. Beckwith, Tight regulation, modulation, and high-level expression by vectors containing the arabinose PBAD promoter. *Jouranl of Bacteriology* **177**, 4121-4130 (1995).
36. L. Imamovic *et al.*, Drug-driven phenotypic convergence supports rational treatment strategies of chronic infections. *Cell* **172**, 121-134.e114 (2018).
37. L. Imamovic, M. O. A. Sommer, Use of collateral sensitivity networks to design drug cycling protocols that avoid resistance development. *Science Translational Medicine* **5**, 204ra132 (2013).
38. R Core Team, "R: A language and environment for statistical computing. R Foundation for Statistical Computing", (The R Foundation, 2018; https://www.r-project.org).
39. M. Thulin, "BAT: an online tool for analysing growth curves", (2018; http://www.mansthulin.se/bat/).
40. D. E. Dykhuizen, Experimental studies of natural selection in bacteria. *Annual Review of Ecology and Systematics* **21**, 373-398 (1990).
41. P. Seideman, O. Beck, S. Eksborg, M. Wennberg, The pharmacokinetics of methotrexate and its 7-hydroxy metabolite in patients with rheumatoid arthritis. *British Journal of Clinical Pharmacology* **35**, 409-412 (1993).
42. J. J. Sacco, J. Botten, F. Macbeth, A. Bagust, P. Clark, The average body surface area of adult cancer patients in the UK: A multicentre retrospective study. *PLOS ONE* **5**, e8933 (2010).
43. S. C. Howard, J. McCormick, C.-H. Pui, R. K. Buddington, R. D. Harvey, Preventing and managing toxicities of high-dose methotrexate. *Oncologist* **21**, 1471-1482 (2016).

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Author contributions: The study was designed by JSG, EGAF, ØS and PJJ. Strain constructions were done by PT and JSG. Experiments were conducted by JSG, EGAF and CIMK. WGS was done using a bioinformatic pipeline designed by JH. Formal data analysis and data visualization was done by JSG. The first draft of the manuscript was written by JSG and revised by PJJ. All authors revised subsequent version and provided constructive feedback on the manuscript. Funding acquisition and resources were provided by PJJ and DIA.

Competing Interests: The authors declare no competing interests. **Data availability:** WGS data are available at NCBI (BioProject PRJNAxxxxxx).

Supplementary materials

Materials and Methods.

Supplementary Text.

Figures S1- S3.

Tables S1-S15.

References 27-43.

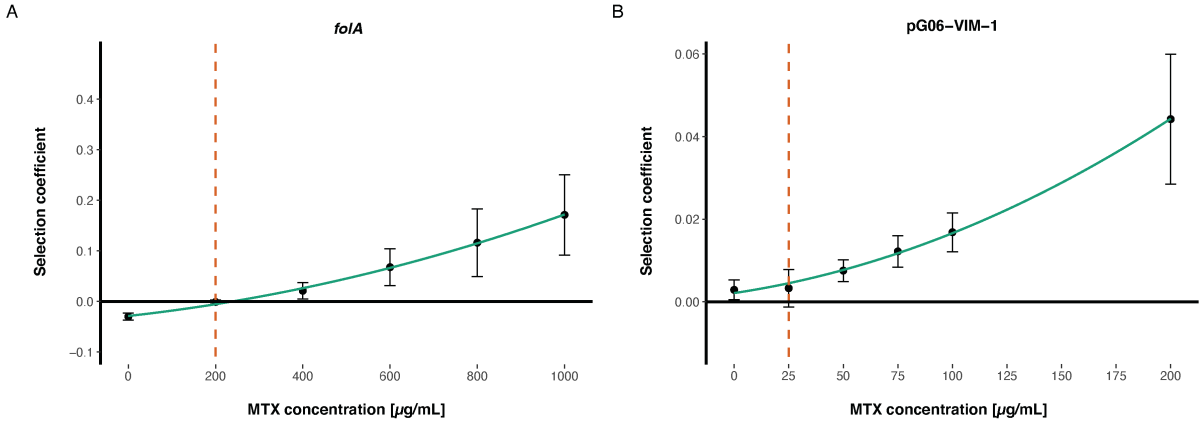

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Figure 1: Selection coefficients as functions of MTX concentrations from competition experiments between TMP^{R} and TMP^{S} isogenic strains. The MSC is defined as the concentration where the selection coefficient equals zero. The MSC for *E. coli* MG1655 harboring **(A)** two chromosomal *folA* mutations (MP18-04 and MP18-07) is set to 200 µg/mL,

280 and **(B)** the MDR pG06-VIM-1 encoding *dfrA1* (MP18-05 and MP18-08) is conservatively set below 25 µg/mL. Dashed lines represent the set MSC, bullets the average selection coefficients and error bars the standard deviations.

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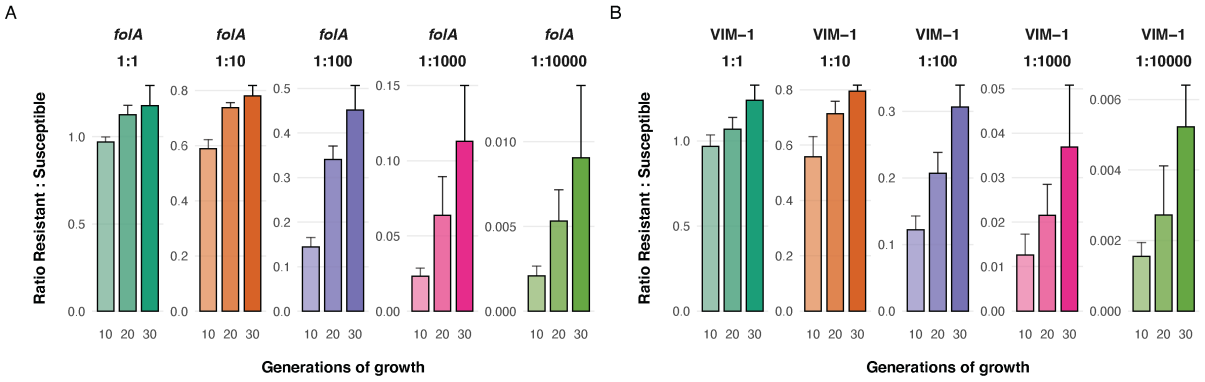

Figure 2: Competition experiments during MXT exposure at different initial frequencies of TMP^{R} strains. The change in TMP^{R}:TMP^{S} ratios over 30 generations of growth at MTX concentrations slightly above the MSCs where *E. coli* MG1655 harbors **(A)** two *folA* mutations

290 (MP18-07), and **(B)** the pG06-VIM-1 plasmid (MP18-08) were competed against a differently tagged, isogenic susceptible strain (DA56507) at 1:1, 1:10^{-2}, 1:10^{-3} and 1:10^{-4} starting ratios. Error bars represent the standard deviation of the average ratio.

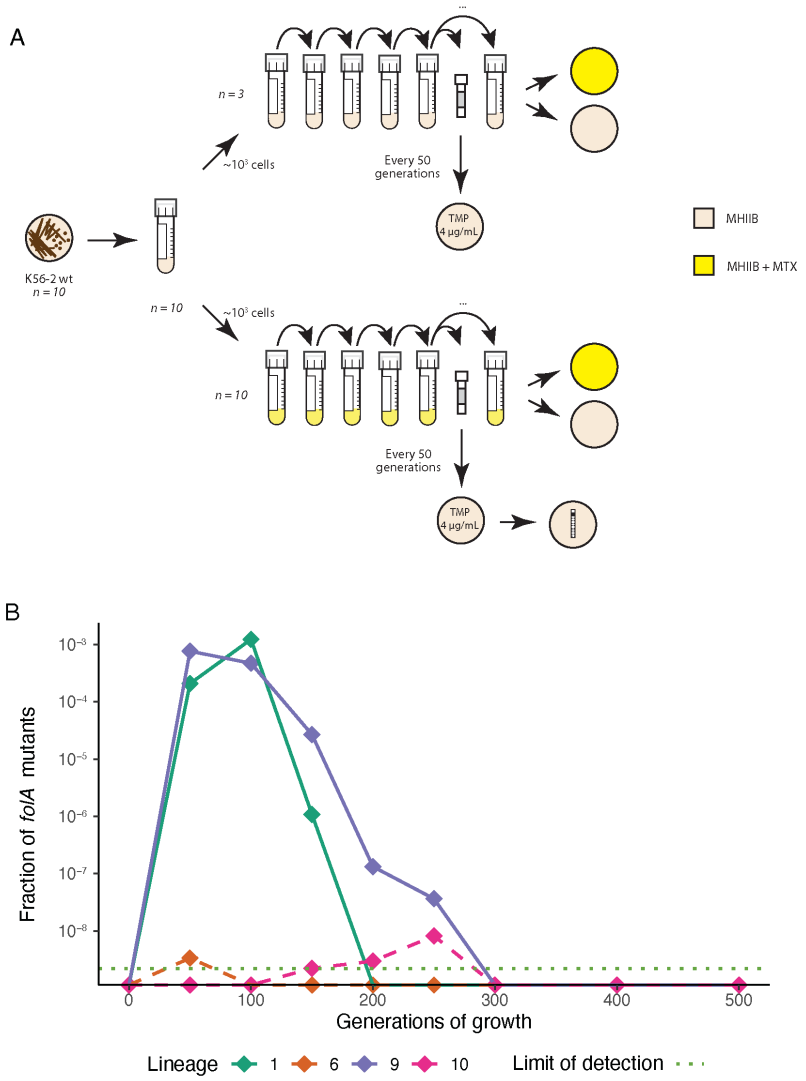

Figure 3: Evolution of TMP^R during MTX exposure for 500 generations. (A) Sub-MIC evolution experimental set-up. Ten biological replicates of K56-2 (MP06-01) were evolved for ~500 generations with 400 µg/mL MTX and three without drug. All lineages were screened for TMP^R every 50 generations. After 500 generations all end-point populations were plated on 32 mg/mL MTX. All populations were able to grow at 32 mg/mL MTX, but not a single clone isolated conferred TMP^R, strongly suggesting that reduced susceptibility to MTX with no cross-resistance to TMP evolved in the endpoint populations. **(B)** Fractions of TMP^R *folA* mutants isolated every 50 generations from the lineages where these were detected. The detection limit of the assay was ~2x10^{-9}. Solid lines represent the two lineages where TMP^R emerged and ascended in frequency whereas dotted lines indicate spontaneous mutants.