Role of dNTP homeostasis in stress adaptation and cell wall biosynthesis of *Mycobacteria*

Tuberculosis (TB) is a communicable disease that is a major cause of ill health and one of the leading causes of death worldwide. Until the coronavirus (COVID-19) pandemic, TB was the leading cause of death from a single infectious agent, ranking above HIV/AIDS[1]. The sustained success of *Mycobacterium tuberculosis* as a pathogen is due to its ability to survive inside macrophages for long periods of time, and its highly unresponsive nature to antibiotics[2]. Furthermore, there is a high incidence of resistance against the few available antituberculotic drugs. Since this phenomenon presents a great bottleneck in efficient TB treatment, understanding the adaptation processes and the stress response of Mycobacteria is in the spotlight of TB research. To gain new insights into the connections between stress tolerance, genetic variability, and the significance of these phenomena in mycobacterial survival strategies, we systematically investigated the effects of currently used antibiotics and environmental stress factors on genome stability, the activation of the DNA repair system and on the dNTP pool using Mycobacterium *smegmatis*, a non-pathogenic model organism for the medically relevant *Mycobacterium* species.

The unique intricate cell wall impermeable for most antibiotics is a specific hallmark of mycobacteria. This cell wall makes mycobacteria highly unresponsive to antibiotics, therefore infections caused by pathogenic mycobacteria are extremely difficult to treat[3]. dTTP, one of the canonical nucleotide-building blocks of DNA, is also used for cell wall biosynthesis in mycobacteria[4]. We hypothesized that the interconnection between DNA and cell wall biosynthesis through dTTP may require synchronization of these processes by regulating dTTP availability. To investigate this, we analyzed growth, morphology, cellular dNTP pool, and possible signs of stress in *Mycobacterium smegmatis* upon perturbation of rhamnose biosynthesis.

Main results of the project

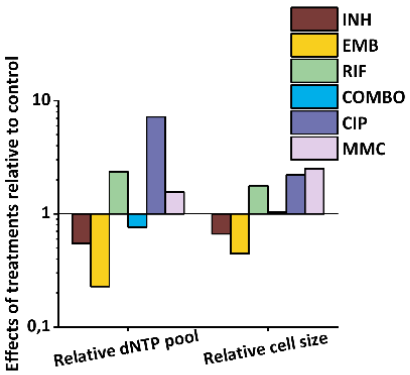
Part 1

We applied various stress conditions on the *Mycobacterium smegmatis* cells to model the effects of the extra- and intracellular environmental stresses of mycobacterial pathogens and the effects of various treatments in mycobacterium infected and treated patients to reveal the possible mechanism of drug resistance development. As this part of the project is still under publication, a more detailed presentation will be applied on this part.

Our main findings are:

We found that the size of dNTP pool is a critical factor in genome stability in *Mycobacterium smegmatis* and also correlating with cell size (Fig. 1A). Cellular dNTP concentration changes were also characteristic to the given treatment. Mycobacterial mutation rates are estimated to be unprecedentedly low and by itself cannot explain the biological diversity, observed in clinical isolates. Therefore, we tested the hypothesis if the remarkable biological diversity is generated by inducible mutagenesis. At one hand, we tested the appearance of stress-induced mutations in the genome, with mutation accumulation assay (Fig. 1B). We also examined the occurance of a CIP tolerant phenotype following incubation with sublethal amount of CIP (Fig. 2). We found that the mycobacterial genome is very stable and the number of newly generated mutations following treatments was very low. The increase of mutation rate was only significant in case of CIP and UV treatment that was the positive control for the experiment.

A



B

Treatment	Mutation rate
Mock (negative control)	5,86E-11
INH	7,95E-11
EMB	<5,86E-11
RIF	4,97E-11
COMBO	2,48E-11
CIP	2,12E-10
MMC	1,06E-10
UV	1,48E-08

Figure 1. Size of the dNTP pool is critical for genome stability maintenance in Mycobacterium smegmatis. A) Relative cellular dNTP content and cell size normalized to untreated control. In consequence of various treatments, dNTP pools changed specifically correlated with cell size. B) Mutation rates measured at different treatments. Mutation rates were elevated only in cases of CIP, MMC and UV treatment shown with red. INH – isoniazid; EMB – ethambuthol; RIF – rifampicine; COMBO – combination treatment of INH, EMB, RIF and pyrazinamide prevalently used in the clinicum; CIP – ciprofloxacin; MMC- mitomycinC.

Our mutation accumulation assay revealed that there is large genetic variance, even in a culture started from a supposedly single cell colony. In response to the given treatment, different sets of enzymes are activated specifically promoting phenotypic resistance or protecting genomic integrity (Fig. 2). A moderate strategy for a bacterium to optimize its fitness in a fluctuating environment is a diversity generated by non-genetical factors (phenotypic heterogenity). Phenotypic resistance (we term it tolerance) development is significant according to our measurements and already existing variants are likely to expand as an adaptation to environmental conditions.

A

	Target	INH	EMB	RIF	COMBO	CIP	MMC
DNA glycosylation	AlkA	2.68	-1.19	2.12	8.58**	-1.71**	-1.48
	Mpg	-2.91	1.05	1.37**	5.55**	-2.68**	-1.46
	MutM1	-1.70	-3.72*	-1.33	4.03**	-1.66	-1.58
	MutY	-3.04	-1.19	-1.12	2.66	-1.39	-1.52
	Nei1	-1.77	-5.73	-1.34	2.62	-1.33	-1.52
	Nei2	-4.25**	-3.28	-1.33	2.03	-1.30	1.37
	Ogt	1.90	1.84	3.19	4.98**	1.31	-1.39
	TagA	-3.43*	-1.11	-1.24	2.22*	4.44**	1.93
	UdgB	-1.22	3.51	1.69	5.43**	1.04	-1.01
	UdgX	-2.04	-2.32*	3.29	1.29	1.18	-1.17
	Ung	-1.46	2.94	-1.01	-1.24	-2.68**	-1.98
Nucleotide excision repair	Mfd	-1.74	1.12	1.38	1.50	3.83**	1.42
	UvrA	-1.04	-1.14	1.39	-1.05	1.98*	1.06
	UvrB	-1.01	1.13	1.45	1.79	5.51**	2.06
	UvrC	-4.08**	-2.13	-2.36	2.55	-1.14	-1.30
	UvrD	-2.09**	-5.76	-1.30	1.99*	2.45**	1.60
dNTP pool sanitization	Dcd:dut	-1.06	1.27	-1.50	1.97	1.69	-1.07
	Dut	-2.22*	-3.39	-1.14	2.31	-1.34	-1.74
	MutT1	-3.30	-2.16	-1.12	1.86	-1.51	-1.15
	MutT2	1.42	-3.22	-2.53	6.08**	-2.59**	-1.66
	MutT3	-1.34	-4.11	-2.55	1.93*	1.06	-1.38
	MutT4	-1.26	1.00	2.15	1.13	1.90	-1.24
	ThyA	-18.96**	-4.95	-2.16	1.67	-2.53**	-1.63
	ThyX	-3.64	-1.90	-2.38	2.41	-1.03	-1.79
DNA synthesis	DinB1	2.45	-3.09	-1.44	2.10	-1.07	1.18
	DinB2	1.10	1.52	1.38	3.53**	12.69**	7.51
	DNA ligase	-1.48	1.70	-1.04	1.85	3.17*	1.99
	DnaE2	2.65	-1.22	-1.52	5.63**	10.49**	8.48**
	PolA	-1.88	1.06	1.68	1.79	3.77**	1.35
Double-strand break repair	AdnA	-1.49	1.20	2.28	1.15	14.12**	7.90*
	LexA	1.12	2.05	-1.18	-1.18	13.34**	6.27
	RecA	-1.04	1.41*	-1.33	-1.41	7.50**	6.69**
	RecX	1.05	1.66	-1.67	1.32	6.23**	5.34*
Endo-nuclease	End	-1.22	1.21	-2.05	2.56*	3.69	2.26**
	NucS	1.57	1.13	-1.08	2.89	1.03	-1.11
	XthA	-1.02	1.48	1.03	3.55**	1.10	-1.75
Peroxidase	AhpC	-1.82	-1.76	-4.38**	1.26	-1.33	-1.4
	KatG1	-3.00**	-4.21	-1.24	-17.17**	1.42	1.02

B

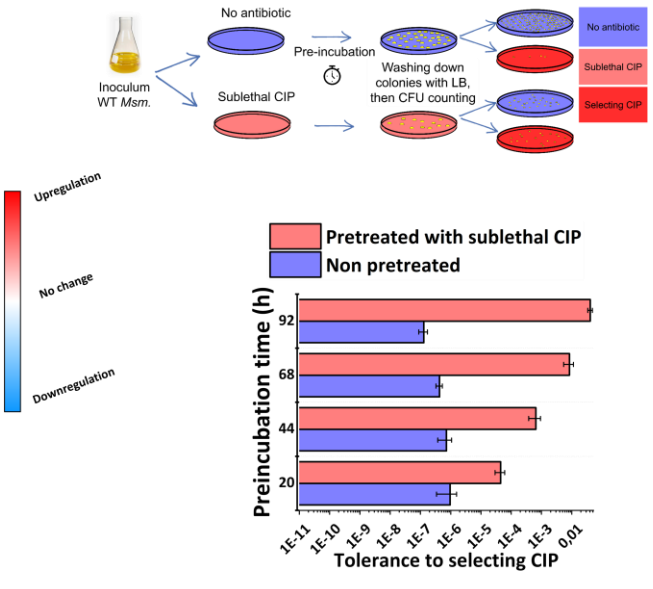


Figure 2. Phenotypic stress response in Mycobacterium smegmatis is a driving force to the fixation of genetic variants. A) Gene expression changes in consequence of different treatments. B) Effect of preceding sublethal CIP treatment on tolerance development against the same drug. Pretreatment augments tolerance in *Mycobacterium smegmatis* for CIP. INH – isoniazid; EMB – ethambuthol; RIF – rifampicine; COMBO – combination treatment of INH, EMB, RIF and pyrazinamide prevalently used in the clinicum; CIP – ciprofloxacin; MMC- mitomycinC.

We found that following short-term exposure to genotoxic stress, the activation of non-genetic cellular factors including e.g. SOS-response or redox potential changes, conveys the observed drug-tolerant phenotypes. We also detected very quick adaptation to selecting amount of CIP when pretreated with sublethal dose of drug. This quick adaptation cannot be explained only with elevated mutation rates. We propose that a possible explanation for this phenomena is that a pre-selection happens against the most susceptible cells in the beginning of the treatment (this is manifested in TB patients as a biphasic kill curve during chemotherapy, with a quick killing of sensitive cells followed by a slow clearance of the remaining ones). Thus the remaining bacteria will be more tolerant, when antibiotic is present. This could allow the fixation of a genetic variant that has some advantage, but otherwise would not spread. More interestingly, harsh long-term exposure to antibiotics known to elicit tolerance in TB treatment did not result in *de novo* adaptive mutations in our experiments. Instead, we observed an expansion of pre-existing genetic variants. These results challenge the currently reigning hypothesis of the development of resistance to antibiotics by microevolution, supporting the model in which the host accumulates a remarkable genetic variation of pathogens during the latent phase of infection and then adaptive selection of certain variants may occur of this pre-existing pool. The long latency and the world-wide high infection rate also suggest that the number of polyclonal infections are underestimated which is in line with our conclusions.

Therefore, these observations together suggest that drug tolerance is formed mainly by phenotypic stress response and rising pre-existing mutations upon the treatments and not by spontaneous mutagenesis. Thus, phenotypic stress response may be the driving force to the fixation of more viable genetic variants.

To fill a longstanding gap in genome metabolism research, we also established dNTPpoolDB, a database that offers access to quantitative data on dNTP pools from a wide range of species, experimental and developmental conditions (https://dntppool.org/). Stimulated by the growing interest in the role of the dNTP pool in physiological and malignant processes, dNTPpoolDB offers a comprehensive collection of dNTP data and pool reconstructions available from a wide range of biological samples[5]. The database comprises quantitative data on the four canonical building blocks of DNA, dATP, dGTP, dCTP and dTTP, as well as exotic dNTPs also incorporated if available. dNTPpoolDB is manually curated by our research group, each entry is verified by a competent annotator. The database was published in NAR this year.

In the first two years of the project, a large amount of quantitative data from dNTP measurements, DNA repair gene expression levels, and mutation rate and spectra data was acquired in function of the various genotoxic treatments. From this part of the project, we planned 3 publication, from which 1 is already published in 2022 (dNTP database paper). Based on first/second line drug treatments and environmental stress factors two other manuscript have written. We will send them for publication soon.

Part 2

In this part of the project, the connections between dNTP homeostasis and cell wall biosynthesis was investigated. We investigated growth, morphology, cellular dNTP pool, and possible signs of cellular stress (cell wall biosynthesis or replication stress) in *Mycobacterium smegmatis* upon perturbation of rhamnose biosynthesis by the overexpression of RmlA. RmlA is a cell wall synthetic enzyme that uses dTTP as the precursor for cross-linking the peptidoglycan with the arabinogalactan layers by a phosphodiester bond in the mycobacterial cell wall[6].

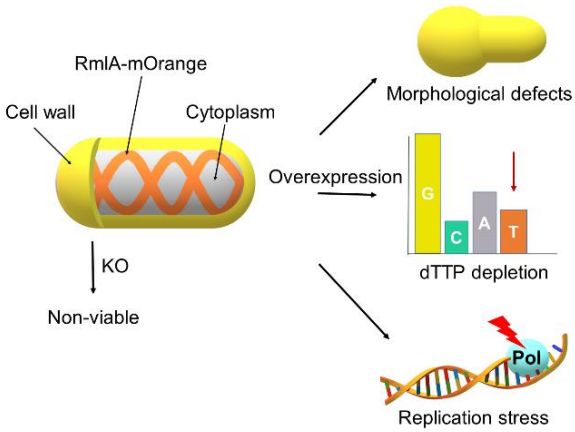


Figure 3. Effects of perturbation of rhamnose biosynthesis in Mycobacterium smegmatis.

We found that RmlA overexpression results in changes in cell morphology, causing cell elongation and disruption of the cylindrical cell shape[7]. We also found that the cellular dTTP pool is reduced by half in RmlA overexpressing cells and this reduced dTTP availability does not restrict cell growth. We observed 2-6-fold increases in the gene expression of selected replication and cell wall biosynthesis stress factors upon RmlA overexpression indicating crosstalk between the two biological process. Using super-resolution microscopy, we found that RmlA, acting to crosslink the nascent layers of the cell wall, localizes throughout the whole cell length in a helical pattern in addition to the cellular pole. Our investigations unveiled that the cylindrical part of the cell may not be as inert as previously thought. The fact that RmlA, acting to crosslink the peptidoglycan and arabinogalactan layers, localizes throughout the whole cell length in a helical pattern strongly suggests that cell wall synthesis occurs not only at the cell poles. Consistent with this, the morphological changes upon RmlA overexpression indicate that RmlA plays a role in determining cell shape driven by a yet unknown mechanism in mycobacteria. These results were published in PlosONE.

Publications:

The effects of mycobacterial RmlA perturbation on cellular dNTP pool, cell morphology, and replication stress in *Mycobacterium smegmatis*. **Hirmondó R**, Horváth Á, Molnár D, Török G, Nguyen L, Tóth J., PLoS One. 2022 Feb 24;17(2):e0263975. doi: 10.1371/journal.pone.0263975.

dNTPpoolDB: a manually curated database of experimentally determined dNTP pools and pool changes in biological samples. Pancsa R, Fichó E, Molnár D, Surányi ÉV, Trombitás T, Füzesi D, Lóczi H, Szijjártó P, **Hirmondó R**, Szabó JE, Tóth J., Nucleic Acids Res. 2022 Jan 7;50(D1):D1508-D1514. doi: 10.1093/nar/gkab910.

Papers under publication:

The roles of phenotypic, and genotypic stress-response of mycobacterial adaptation. Molnár D, Surányi ÉV, Trombitás T, Füzesi D, **Hirmondó R** and Tóth J., manuscript is under submission

Investigation of the mutator effects of various genotoxic stresses provides insight into the mechanism of drug resistance development in Mycobacteria. **Hirmondó R**, Surányi ÉV, Molnár D, Trombitás T, Füzesi D, Tóth J., manuscript is under construction

Conference attendance in the project period:

2018. 09. 02-05. FEBS3+ conference, Siófok. Poster presentation: **R. Hirmondó**, B. S. Mébold, J. Tóth: Investigation of the specific role of dTTP homeostasis in mycobacterial cell wall biosynthesis

2019. 03. 29-31. Hungarian Molecular Life Sciences, Eger. Poster presentation: **R. Hirmondó**, É. V. Surányi, D. Molnár, Á. Horváth, B. G. Vértessy, J. Tóth: Investigation of the mutator effects of various genotoxic stresses provides insight into the mechanism of drug resistance development in Mycobacteria

2019. 06. 09-12. EMBO Workshop: Bacterial cell division: Closing the gap, Lund, Sweden. Poster presentation: **R. Hirmondó**, Á. Horváth, J. Tóth: Cellular dTTP homeostasis provides a possible checkpoint between replication and cell wall biosynthesis in mycobacteria

2019. 08. 27- 09. 07. FEMS Summer School for Postdocs: Biological Robustness: Evolution of Bacterial Resistance to Death. Split, Croatia. Poster presentation: **R. Hirmondó**, É. V. Surányi, D. Molnár, Á. Horváth, T. Trombitás, D. Füzesi, N. Gálik, H. Lóczi, B. G. Vértessy, J. Tóth: Investigation of the mutator effects of various genotoxic stresses provides insight into the mechanism of drug resistance development in Mycobacteria. (winning "Best project award")

2019. 09. 26-27. 10th Central European Genome Stability and Dynamics Meeting, Bratislava, Slovakia. Poster: **R. Hirmondó**, É. V. Surányi, D. Molnár, Á. Horváth, T. Trombitás, D. Füzesi, N. Gálik, H. Lóczi, B. G. Vértessy, J. Tóth: Genotoxic stress conditions associated with mycobacterial life-style has important role in drug resistance development

2020. 10. 28-30. FEMS Online Conference on Microbiology, Selected for oral presentation; **R. Hirmondó**, D. Molnár, ÉV. Surányi, D. Füzesi, J. Tóth: Investigation of the mutator effects of currently used TB drugs provides insight into the mechanism of drug resistance development in Mycobacteria

2020. 11. 12. FIBOK (National Conference of Young Biotechnologists), Online conference, oral presentation. **R. Hirmondó**, É. V. Surányi, D. Molnár, Á. Horváth, T. Trombitás, D. Füzesi, N. Gálik, H. Lóczi, B. G. Vértessy, J. Tóth: Betekintés a gyógyszerrezisztencia kialakulásába – a TB gyógyszerek mutátor hatásainak vizsgálata

2021. 07. 3-8., 45th FEBS Congress, Ljubjana, poster presentation. **R. Hirmondó**, ÉV. Surányi, D. Molnár, T. Trombitás, D. Füzesi, J. Tóth: Investigation of the mutator effects of various genotoxic stresses provides insight into the mechanism of drug resistance development in Mycobacteria (the poster was awarded a FEBS Open Bio poster prize)

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7. Hirmondó R, Horváth Á, Molnár D, Török G, Nguyen L, Tóth J. The effects of mycobacterial RmlA perturbation on cellular dNTP pool, cell morphology, and replication stress in Mycobacterium smegmatis. PLoS One. 2022;17. doi:10.1371/JOURNAL.PONE.0263975