Theses of the PhD dissertation entitled

Distinct control of dNTP biosynthesis and genome integrity maintenance by dUTPases

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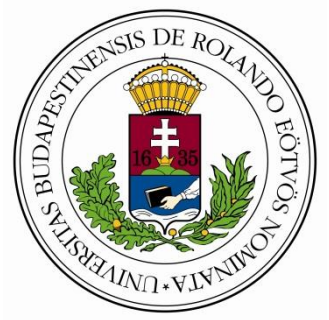

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1 Introduction

Proper control of the intracellular concentration of deoxyribonucleoside-5-triphosphates (dNTPs), the building blocks of DNA, is critically important for efficient and high-fidelity DNA replication and genomic stability [1]. Three of the four canonical dNTPs are synthesized from their respective ribonucleoside diphosphate (NDP) counterparts [2]. The direct precursor for dTTP, however, is missing from the ribonucleoside pool and is synthesized via separate routes.

The *de novo* synthesis of dTTP occurs through uracil base-containing precursors: dUMP is the direct input into the thymidylate synthase reaction. In most organisms, the main dUMP supply arises from the deamination of a cytosine deoxyribonucleotide (dCMP or dCTP) while the dephosphorylation of UDP is considered to be a minor supplement [3]. When cytosine deamination occurs at the triphosphate level, the resulting dUTP is then converted into dUMP. The enzymes that catalyze these conversions belong to the dUTPase superfamily comprising dCTP deaminase (Dcd), dUTPase (Dut) and the bifunctional dCTP deaminase/dUTPase (Dcd:dut). In addition to dUMP production, the dUTPase reaction also serves to eliminate excess dUTP to prevent uracil incorporation into DNA in place of thymine [4]. Although not mutagenic when replacing thymine, the uracil in DNA is considered an error and induces uracil-excision repair mechanisms [5]. In high dUTP/dTTP ratios, however, DNA polymerases keep reincorporating dUTP and the repair process becomes overwhelmed eventually resulting in cell death. Recently, novel functions of Dut emerged in gene expression regulation as well [6,7]. It was shown that a pathogenicity island repressor protein in *Staphylococcus aureus*, *Stl* is capable of interact with the helper phage dUTPase and the repressor activity can be inhibited by binding to it [7]. This interaction was further characterized, and *Stl* proved to be also a potent protein inhibitor of *Staphylococcus aureus* phage *Φ11* dUTPase [6].

In summary, Dut catalyzes the break-down of dUTP to dUMP and with this action it takes part in i) dTTP biosynthesis, ii) the maintenance of low dUTP/dTTP ratio to prevent uracil incorporation into DNA, iii) interactions with regulatory proteins. The various roles now attributed to Dut and the large amount of unselective knock-out and knock-down data on the dUTPase superfamily enzymes create a confusing picture of the contribution of Dut to the physiological processes in which it may be involved. As dTTP biosynthesis is an essential process and a major target in several drug therapies, it is important to determine whether Dut is a key contributing enzyme.

2 Aims

We therefore set-out to dissect the contributions of dUTP-hydrolyzing enzymes, Dut and Dcd:dut, to dTTP biosynthesis and to the prevention of DNA uracilation. For this reason, we looked for a simple model in which the obligatory dTTP precursor, dUMP, is produced exclusively by Dut and Dcd:dut in lack of salvage pathways and dCMP deamination. This favorable set of conditions naturally occurs in the genus *Mycobacteria*. Due to the exclusive biosynthetic role of dUTPases in these organisms, they present potential targets for drug development, as well.

1. We wished to create a dUTPase knock-out bacterial strain in *M. smegmatis* to investigate the physiological role of dUTPase. We also wanted to construct our point mutant dUTPase coding *M. smegmatis* strains using the Dut KO strain by complementing the lethal gene disruption with the mutant enzymes.

2. We aimed to create *M. tuberculosis* dUTPase mutant proteins in which the enzyme activity is gradually tuned down. We designed *M. smegmatis* strains carrying the same Dut mutations and also included an inactive *dcd:dut* mutant strain into the experiments to reveal the physiologic linkage between dTTP biosynthesis and prevention of uracil misincorporation.

3. To investigate if the recently found dUTPase inhibitor protein, *Stl* may also prove to be a potent inhibitor of the mycobacterial dUTPase, we designed *in vitro* binding and inhibition experiments with *Mycobacterium tuberculosis* dUTPase. The positive outcome of these experiments inspired the in vivo study of Stl expression in the *M. smegmatis* cell.

3 Materials & Methods

On the basis of our previous investigations on the human and *Echerichia coli* (*E.coli)* dUTPases [8,9], we planned and created several mutants of the *M. tuberculosis* Dut enzyme by site-directed mutagenesis. These mutants were chosen to represent enzymatic activity loss from one to a few orders of magnitude. To investigate the effect of dUTPase activity loss in the living cell we created these *dut* mutations and a *dcd:dut* mutation within the genome of *M. smegmatis*. We established a method in which the disruption of the endogenous gene was rescued by a functional copy of the gene (either with a mutant copy) inserted into the genome. Viability of the strains was checked by following optical density of the liquid culture growing under stress-free conditions.

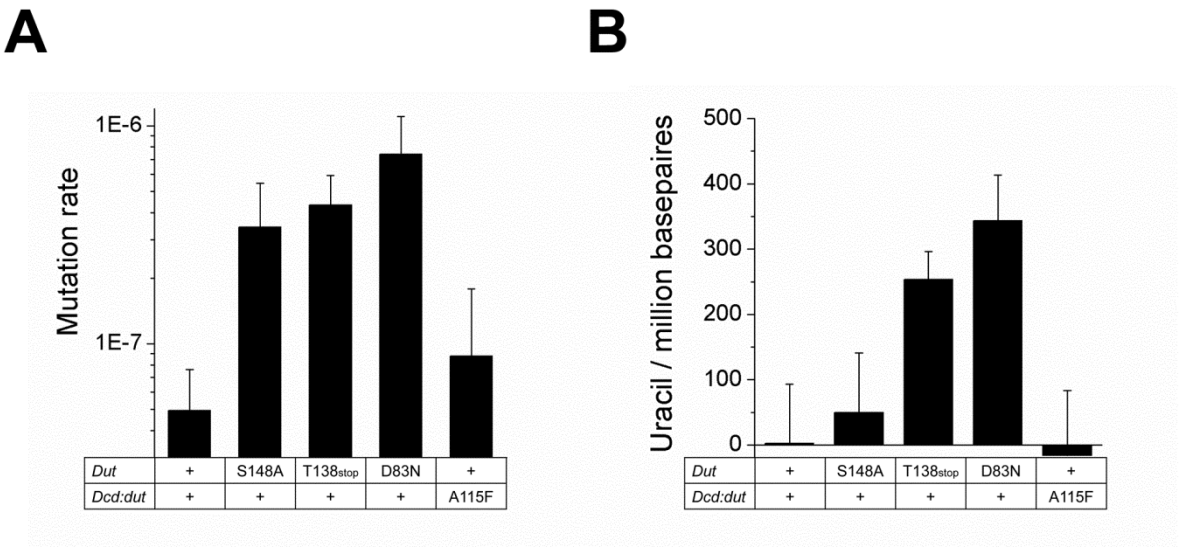
Spontaneous mutation rate against rifampicine was measured according to David et. al. [10]. To investigate mutational spectrum, we sequenced 1000 bp of the *M. smegmatis rpoB* gene responsible for rifampicine resistance including the mutation hot-spot region of DNA isolated from rifampicine resistant wt, *dut* and *dcd:dut* mutant strains. Genomic uracil content of the mutant strains was measured by a q-PCR based method [11]. Pyrimidine nucleotide concentrations (dUTP, dTTP and dCTP) in the mutant *M. smegmatis* strains were measured by a PCR-based method [12]. This method is based on the incorporation of radiolabeled dATP into a nucleotide-specific template limited by the concentration of the quantifiable dNTP.

To investigate whether the recently found dUTPase inhibitor protein, Stl may also bind to mycobacterial dUTPase, we carried out native gel electrophoresis experiments. We designed steady-state activity measurements with mtDUT to investigate whether Stl can also inhibit the mycobacterial enzyme. We tested the inhibition on the wild type enzyme and on the quasi wild type variant (H145W) containing a tryptophan substitution at the active site. To investigate whether Stl can inhibit the dUTPase function in the Mycobacterium cell, we constructed *M. smegmatis* strains that expressed the Stl protein either constantly or in an inducible manner. Pyrimidine nucleotide concentrations (dUTP, dTTP and dCTP) were measured by a PCR-based method [12] applied earlier on the *dut* mutants. Colony forming capacity of the strain expressing Stl was measured after induction of Stl expression.

4 Results & Discussion

dTTP biosynthesis and the exclusion of uracil from DNA for genomic stability are linked through the dUTPase reaction. The hydrolysis product dUMP has exclusive biosynthetic role in dTTP biosynthesis while the elimination of excess dUTP prevents DNA uracilation. We combined enzymology and genetics to investigate this potential double role in *Mycobacterium smegmatis* beneficially conferring two dUTPases and no alternative ways to produce dUMP.

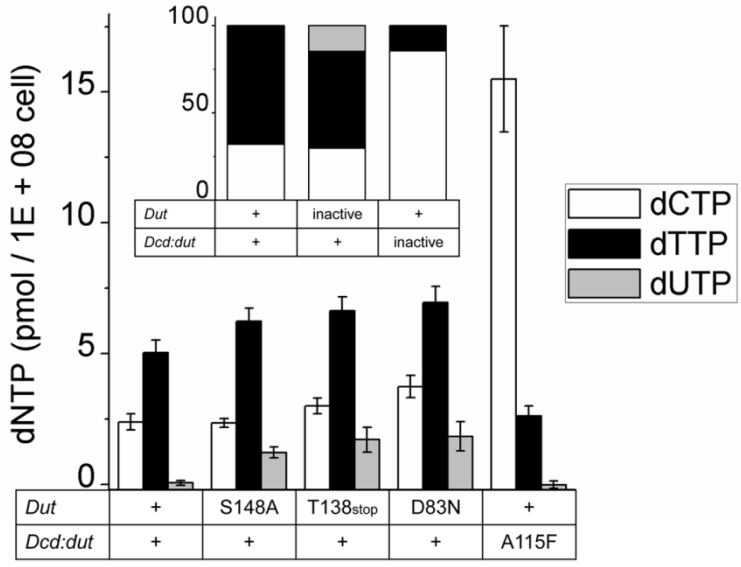
- We constructed mutant bacterial strains in which *dut* activity was gradually tuned down.
- We detected that the mutation rate and genomic uracil content increased in correlation with the engineered *in vitro* activity-loss while the mutational spectrum and the dNTP balance remained normal in the *dut* mutant strains.
- In contrast, mutants carrying inactive *dcd:dut* exhibited a highly perturbed dNTP balance and markedly changed mutational spectrum.



1. Figure ***A.)*** *Mutation rates of mutant M. smegmatis strains calculated from three independent strains from each mutant; means ± SE are plotted. Note that mutation rates of dut mutant strains directly correlate with in vitro activity-loss of the mutant dUTPase enzyme.* ***B.)*** *Genomic uracil content of the mutant strains compared to wt strain. Uracil contents were calculated from three independent strains from each mutant and normalized to wt strain; means ± SE are plotted. Note that genomic uracil content of dut mutant strains directly correlate with in vitro activity-loss of the mutant dUTPase enzyme.*

- We show that dUTPase (*dut*) and the bifunctional dCTP deaminase/dUTPase (*dcd:dut*) sharing the same core structure and reaction product seem to be adapted to the distinct roles of genome integrity maintenance and dNTP pool balancing.

- We suggest that de-coupling of dNTP regulation and uracil elimination is advantegous for maintaining genome integrity.



2. Figure *Pyrimidyne nucleotide pool of mutant strains. dTTP, dCTP and dUTP were measured with a pcr-based method in all mutant strains. Mutant enzymes are indicated, '+' mean wt enzyme. Three parallel strain were used from each mutation in the experiments; means ± SE are plotted. The percentage of pyrimidine nucleotides were plotted in the inset of the figure.*

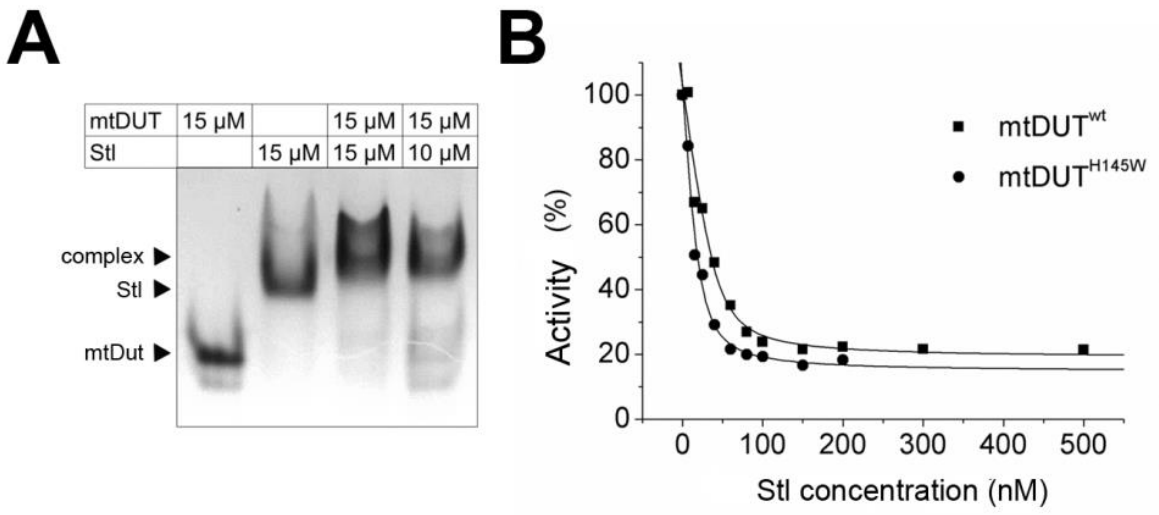
The various roles now attributed to Dut and the large amount of unselective knock-out and knock-down data on the dUTPase superfamily enzymes creates a confusing picture of the contribution of Dut to the physiological processes in which it may be involved. In addition, dTMP production by salvage pathways usually interfere with effect of the dUTPase knock-out / knock-down experiments. We therefore set-out to dissect the contributions of dUTP-hydrolyzing enzymes, Dut and Dcd:dut, to dTTP biosynthesis and to the prevention of DNA uracilation. For this reason, we looked for a simple model in which the obligatory dTTP precursor, dUMP, is produced exclusively by Dut and Dcd:dut in lack of salvage pathways and dCMP deamination. This favorable set of conditions naturally occurs in the genus *Mycobacteria.* Therefore, we combined enzymology with genetics to address the role of dUTPase activity within the cell. We found that our model organism, *M. smegmatis*, ultimately depends on dUTPase activity to produce dTTP. Our results suggest, however, that

dTTP biosynthesis is mainly under the control of the Dcd:dut enzyme despite it is reported to be much less efficient dUTPase than Dut. This suggests that the mechanistic difference between the two dUTPase enzymes is more important than simply is their catalytic efficiency. Moreover, citozine deaminase enzymes (Dcd, Dcd:dut, Dctd) are able to bind dTTP which inhibits their activity. This negative feedback inhibition allows for the regulation of the cellular dCTP:dTTP concentration ratio. Dut, however, can only accommodate dUTP and does not show any allosteric features.

Our results also indicate the potential of dUTPase as a target for antitubercular drugs and identify a genus-specific surface loop on the enzyme as a selective target. We also show that mycobacterial Dut may have an unidentified moonlighting function associated to the genus specific loop motif.

- We demonstrated that mutant dUTPase gene lacking the genus-specific loop results in lethality.
- We also showed that deletion of the mycobacteria-specific loop has no major effect on dUTPase enzymatic properties *in vitro* and thus a yet to be identified loop-specific function seems to be essential (not enzyme activity of Dut) within the bacterial cell context.

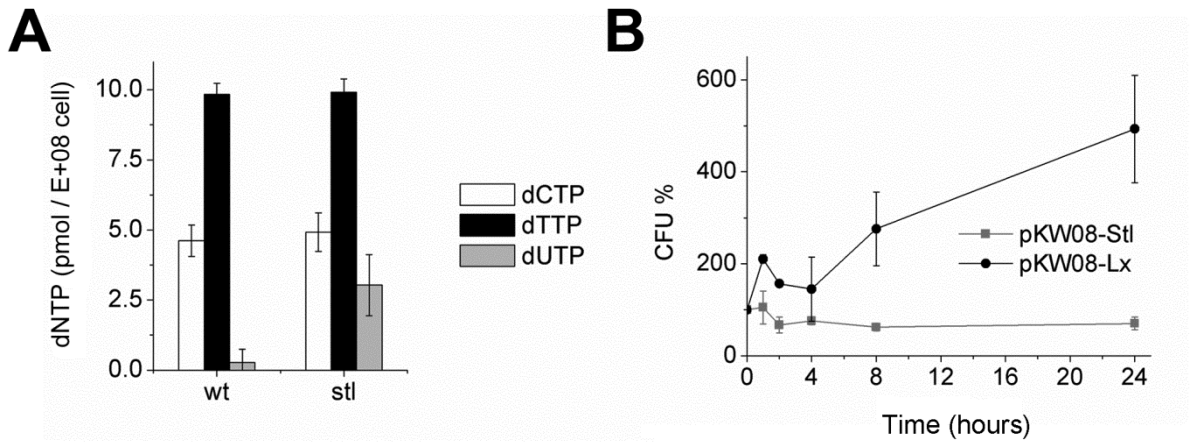
We also reported that a *Staphylococcus* pathogenicity island repressor protein called $Stl_{SaPIbov1}$(Stl) exhibits potent dUTPase inhibition in *Mycobacteria*.



3. Figure Stl forms a stable complex and inhibits Mycobacterium tuberculosis dUTPase in vitro
A.) *Native gel electrophoresis experiment indicates stable complex formation between Stl and Mtb dUTPase. Species and concentrations given in monomers are indicated.* ***B.)*** *Representative*

measurements of Stl's inhibitory effect on mtDUT^{WT} and on mtDUT^{H145W} activity. To ensure comparability data were normalized to the uninhibited activity of the two dUTPase variants. The concentration of dUTPases and dUTP were 50 nM and 30 μM, respectively. Data were fitted using the quadratic binding equation to yield apparent K_i = 5.5 ± 4.6 nM for mtDUT^{WT} and apparent K_i = 4.4 ± 2.8 nM for mtDUT^{H145W}. The total amplitudes of the activity change were 83.8 ± 6.7 % for mtDUT^{WT} and 87.1 ± 5.8 % for mtDUT^{H145W}, respectively.

- We demonstrated that the *Staphylococcus aureus* Stl and the *Mycobacterium tuberculosis* dUTPase form a stable complex and that in this complex, the enzymatic activity of dUTPase is strongly inhibited.
- We also found that the expression of the Stl protein in *Mycobacterium smegmatis* led to highly increased cellular dUTP levels in the mycobacterial cell, this effect being in agreement with its dUTPase inhibítory role.
- In addition, Stl expression in *M. smegmatis* drastically decreased colony forming ability, as well, indicating significant perturbation of the phenotype.
- To our knowledge, this is the first indication of a cross-species inhibítor protein for any dUTPase.



4. Figure ***A.)*** *Pyrimidine nucleotide pool data measured in Mycobacteria either expressing or not expressing Stl protein. dTTP, dCTP and dUTP were measured with a pcr-based method. dUTPase treatment allows to differentiate between dUTP and dTTP. dTTP values shown are corrected with dUTP level measured in the sample. Three parallel strain were used in the experiments from both Stl expressing pGem-int-Stl transformed bacteria (labeled as 'stl') and both empty vector transformed bacterium (labeled as 'wt'). Means±SE are plotted.* ***B.)*** *Colony-forming unit (CFU) counts after various period of induction of Stl expression. CFU counts were normalized to t=0. Three parallel strains were used in the experiments; means ± SE are plotted.*

Therefore, we propose that Stl can be considered to be a cross-species dUTPase inhibítor and may be used as an important reagent in dUTPase inhibition experiments either *in vitro/in situ* or *in vivo.*

5 References

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6 List of publications

6.1 Publications related to the doctoral thesis

6.1.1 Peer-reviewed publications

Pécsi Ildikó, **Hirmondó Rita**, Amanda C. Brown, Lopata Anna, Tanya Parish, Vértessy G. Beáta, & Tóth Judit (2012). The dUTPase enzyme is essential in Mycobacterium smegmatis. PloS One, 7(5), e37461. doi:10.1371/journal.pone.0037461

Hirmondó Rita, Szabó Judit Eszter, Nyíri Kinga, Tarjányi Szilvia, Dobrotka Paula, Tóth Judit, & Vértessy G. Beáta (2015). Cross-species inhibition of dUTPase via the Staphylococcal Stl protein perturbs dNTP pool and colony formation in Mycobacterium. DNA Repair. doi:10.1016/j.dnarep.2015.03.005

6.1.2 Publications under publication at peer-reviewed journals (*equal contributions)

Hirmondó Rita*, Lopata Anna*, Vértessy G. Beáta, & Tóth Judit. Distinct control of dNTP biosynthesis and genome integrity maintenance by dUTPases. NAR, under review

6.2 Other publications

Könnyű Balázs, Kashif S. Sadiq, Turányi Tamás, **Hirmondó Rita**, Barbara Müller, Hans-Georg Kräusslich, Peter V. Coveney, & Müller Viktor. (2013). Gag-Pol processing during HIV-1 virion maturation: a systems biology approach. PLoS Computational Biology, 9(6), e1003103. doi:10.1371/journal.pcbi.1003103

6.3 Conference proceedings

Hungarian Molecular Life Sciences 2013 (oral presentation, Siófok, 2013) Hirmondó R., Lopata A., Pécsi I., Vértessy B. and Tóth J.: *In vivo* enzymology: distinct control of dTTP biosynthesis and dUTP elimination

EMBO Conference on Microbiology after the genomics revolution: Genomes 2014 (poster presentation, Pasteur Institute, Paris, France, 2014) Hirmondó R., Lopata A., Pécsi I., Vértessy B., and Tóth J.: Distinct control of dTTP biosynthesis and dUTP elimination. (Poster presentation)

EMBO|EMBL Symposium: New Approaches and Concepts in Microbiology (poster presentation, EMBL Heidelberg, Germany, 2013) R. Hirmondó, A. Lopata, B.G. Vértessy and J. Tóth: Distinct control of dTTP biosynthesis and dUTP elimination in *Mycobacterium smegmatis*