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Cross-species inhibition of dUTPase *via* the *Staphylococcal* Stl protein perturbs dNTP pool and colony formation in *Mycobacterium*

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ABSTRACT

Proteins responsible for the integrity of the genome are often used targets in drug therapies against various diseases. The inhibitors of these proteins are also important to study the pathways in genome integrity maintenance. A prominent example is Ugi, a well known cross-species inhibitor protein of the enzyme uracil-DNA glycosylase, responsible for uracil excision from DNA. Here, we report that a *Staphylococcus* pathogenicity island repressor protein called $Stl_{SaPIbov1}$ (Stl) exhibits potent dUTPase inhibition in *Mycobacteria*. To our knowledge, this is the first indication of a cross-species inhibitor protein for any dUTPase. We demonstrate 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 inhibitory role. In addition, Stl expression in *M. smegmatis* drastically decreased colony forming ability, as well, indicating significant perturbation of the phenotype. Therefore, we propose that Stl can be considered to be a cross-species dUTPase inhibitor and may be used as an important reagent in dUTPase inhibition experiments either *in vitro*/*in situ* or *in vivo*.

1. Introduction

Proper control of the intracellular concentration of dNTPs is critically important for efficient and faithful DNA replication and genomic stability [1–3]. In fact, dNTP pool imbalances often cause mutator effects [4–7]. Due to the intriguing lack of a functional mismatch repair system in *Mycobacteria*, with potential important biological implications with respect to the evolution of mycobacterial genomes, mutator effects of nucleotide pool imbalances may have an increased significance [8]. The absence of mismatch repair in *Mycobacteria* can also indicate an elevated importance for the remaining DNA damage recognition and repair pathways in maintaining genomic integrity. These pathways include double-strand break repair as well as the preventive and the base excision repair (BER) mechanisms. Interestingly, *Mycobacteria* have evolved remarkable redundancy in their BER system. For example, *M. tuberculosis* harbors two MutM homologues responsible for excision of 8-oxo-guanin [9] and also two uracil DNA glycosylases [10]. Both MutM and uracil DNA-glycosylases are important in oxidative stress response.

Preventive DNA repair relies on dNTP pool sanitizing pathways, among which dUTPase enzymes catalyze pyrophosphorolysis of dUTP generating dUMP and inorganic pyrophosphate [3,11,12]. This reaction is of key importance since dUTP is constantly produced in the pyrimidine biosynthesis pathways and most (including mycobacterial) DNA polymerases readily incorporate dUMP into DNA as a dTMP replacement if the cellular dUTP pool is elevated. DNA uracilation under high dUTP pool conditions will lead to hyperactive futile cycles of base-excision repair and decreases viability by disrupting genome integrity [13]. To prevent DNA uracilation, dUTPase is required to eliminate excess dUTP. In addition, the product dUMP is the precursor for dTTP biosynthesis. The homotrimer dUTPase enzymes have three active sites, each constituted by conserved sequence motifs (motif I–V) from all three subunits [14–18]. In mycobacterial genomes, a

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bifunctional dCTP deaminase:dUTPase is additionally encoded [19]. This enzyme shows a very similar homotrimeric fold and catalyses both the dCTP deamination reaction and the triphosphate hydrolysis of the resulting dUTP, directly producing dUMP from dCTP. However, the efficiency of the triphosphate hydrolysis by the bifunctional enzyme is several hundred fold less [19] than that of the monofunctional trimeric dUTPase [20]. The monofunctional dUTPase (*dut*) is essential in *Mycobacteria* [21–23]. In contrast, earlier mutagenesis studies found that the presence of the bifunctional dCTP deaminase:dUTPase enzyme is dispensable for growth in *M. tuberculosis* [21,22].

The *Mycobacteria* genus comprises highly challenging pathogens including *M. tuberculosis* and *Mycobacterium leprae*, the causative agents of tuberculosis and leprae, respectively. These pathogens still remain a public health problem worldwide despite extensive research and drug development efforts. Since dUTPase plays an important role in genom integrity maintenance and is essential in *M. tuberculosis*, it may serve as an effective drug target for developing novel drug candidates [20,24–26]. In addition to attempts in identification of small molecule inhibitors, highly potent proteinaceous inhibitors can be also very useful in studying the role and function of the target protein in the living cell. Proteinaceous inhibitors can be expressed under different conditions and provide versatile and revealing tools for the investigation of various cellular pathways. In this respect, it is worthwhile to point out that the function of the uracil-DNA glycosylase (Ung) enzyme, responsible for uracil excision from DNA, can be readily inhibited by a widely characterized and generally used inhibitor protein (Ugi) in different *in vitro*/*in vivo* experiments [27–29]. However, as of present, there is no such report on a general inhibitor protein for dUTPases. Although the presence of potential dUTPase inhibitor proteins in *Bacillus subtilis* and *Drosophila melanogaster* was suggested in the early literature [30,31], till now none of these suggestions could be confirmed at the molecular level. However, it was recently shown that a pathogenicity island repressor protein in *Staphylococcus aureus*, $Stl_{SaPIbov1}$ (Stl), is capable of interacting with the helper phage dUTPase and that the Stl repressor activity is inhibited in the complex [32,33]. Detailed quantitative characterization of this interaction also revealed that the Stl repressor is a highly potent protein inhibitor of the *S. aureus* phage *Φ11* dUTPase, as well [33].

Here, we aimed to investigate if Stl acts as a dUTPase inhibitor in a highly unrelated species, we designed *in vitro* binding and inhibition experiments with *M. tuberculosis* dUTPase (mtDUT). The positive outcome of these experiments inspired the *in vivo* study of Stl expression in the *Mycobacterium smegmatis* (*M. smegmatis*) cell.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

M. smegmatis mc²155 was grown in Lemco medium (broth) or with the addition of 15 g L^{-1} Bacto agar (solid). Gentamycin was added at 10 μg/mL and hygromycin B at 100 μg/mL final concentration.

2.2. Expression and purification of Stl protein

For expression of Stl, *Escherichia coli* BL21(DE3) pLysS cells transformed with pGEX-4T-1vector containing Stl-GST fusion protein [33] were propagated in 500 mL LB to an OD_{600} of 0.6, then the culture was cooled to 30 °C and than induced with 0.5 mM isopropyl-β-D-thiogalactoside. After induction, the cell cultures were grown for a further 4 h at 30 °C. Finally the cells were harvested by centrifugation. Subsequent manipulations were carried out on ice. For purification of Stl protein, cell pellets were solubilized in 15 mL of buffer A (PBS (pH 7.3), 5 mM $MgCl_2$) supplemented with 400 mM NaCl, 2 mM dithiothreitol (DTT), 1% Triton X-100, 2 μg/mL RNase and Dnase and one tablet of cOmplete ULTRA Tablets, Mini, EDTA-free protease inhibitor. Cell suspensions were stirred for 10 min, sonicated (60 s, 4 times), and centrifuged (16,000 × *g* for 30 min). Supernatant was diluted in buffer A to contain 200 mM NaCl and loaded on a preequlibrated benchtop glutathion-agarose affinity-chromatography column. The column was washed with ten volumes of buffer A (200 mM NaCl). After that, 80 cleavage unit thrombin was added to perform on-column cleavage for the removal of glutathion-S-transferase tag. After overnight cleavage, Stl protein was eluted from the column (at >95% purity, as estimated from SDS-PAGE). Stl protein was stored in 400 mM NaCl to obtain suitable protein stability.

2.3. Expression and purification of dUTPase

Recombinant dUTPases (wild type *M. tuberculosis* dUTPase ($mtDUT^{wt}$) and the point mutant enzyme conferring a single Trp in the active site ($mtDUT^{H145W}$)) carrying an N-terminal hexa-His tag were expressed from pET19-b vector in *E. coli* BL21(DE3) pLysS cells [20]. For protein overexpression, the cells were grown to an OD_{600} of 0.4, treated with 0.5 mM isopropyl-b-D-thiogalactopyranoside at 37 °C for 3 h. Protein purification was carried out as described previously [20]. The final supernatant after cell extraction was loaded on a Ni-NTA column (Novagen) and purified according to the Novagen protocol. The purity of the protein preparation was analyzed by SDS-PAGE.

2.4. Steady-state colorimetric dUTPase assay

Protons released in the dUTPase reaction were detected by phenol red pH indicator in 1 mM HEPES pH 7.5 buffer also containing 150 mM KCl, 40 μM phenol red (Merck) and 5 mM $MgCl_2$ [34–37]. A Specord 200 (Analytic Jena, Germany) spectrophotometer and 10 mm path length thermostated cuvettes were used at 20 °C. Absorbance was recorded at 559 nm. Initial velocity was determined from the slope of the first 10% of the progress curve. For the determination of the apparent K_i of Stl inhibition reaction mixtures contained 50 nM enzyme and varying concentrations of Stl. Reaction was started with the addition of 30 μM dUTP after 5 min preincubation of the two proteins. Stl inhibition data were fitted to the quadratic binding equation describing 1:1 stoichiometry for the dissociation equilibrium with no cooperativity:

$$y = s + \frac{A[(c + x + K_i) - \sqrt{(c + x + K_i) - 4cx}]}{2c}, \quad (1)$$

where *x* is the Stl concentration and *y* is the relative enzyme activity (normalized to the uninhibited activity), $s = y$ at $x = 0$, *A* is the total amplitude of the activity change, c is the enzyme concentration and K_i is the apparent inhibition constant of Stl inhibition.

For the determination of Michaelis–Menten parameters of dUTPase in the absence and presence of Stl reaction mixtures contained 50 nM enzyme, 150 nM Stl and varying concentration of dUTP. Reaction was started with the addition of dUTP after 5 min preincubation of the two proteins. Michaelis–Menten equation was fitted to the initial velocity data. Data were analyzed in Origin 8.5 (OriginLab Corp., Northampton, MA).

2.5. Native gel electrophoresis

Native gel electrophoresis was performed in 8% polyacrylamide gel, which was pre-equilibrated with pH 8.7 Tris–HCl buffer for 30 min with constant voltages of 100 V. Then 20 μL of samples were added to each well and the electrophoresis was performed

for 2.5 h at 150 V. Sample concentrations are indicated. During electrophoresis the apparatus was cooled on ice. The gel was stained with Comassie-Brilliant Blue dye.

2.6. Construction of Stl expressing M. smegmatis strains

The strong promoter of Kanamycin gene from p2NIL plasmid [38] was PCR-amplified and cloned into the vector pGem-T-easy (Promega). The Gm-Int HindIII cassette from the pUC-Gm-Int plasmid [38] was introduced into the resulting construct to yield the integrating vectorpGem-int-empty (used as control). The Stl coding region with AU1-tag was PCR-amplified from pGEX-4T-1 expression vector (generated in [33]) and cloned after the promoter (ending in NheI restriction site) in the vector pGem-int-empty with NheI restriction sites to get pGem-int-Stl. Successful cloning was verified with sequencing of the appropriate region of the plasmid. 0.5–0.5 μg of pGem-int-Stl and pGem-int-empty (used as control) were electroporated into electrocompetent wt *M. smegmatis* strain. Three parallel strains from each were chosen and used in the experiments. Primers used for cloning are compiled in Table S1.

2.7. Construction of inducible Stl expressing M. smegmatis strains

The Stl coding region with AU1-tag from pGEX-4T-1 was pcr-amplified and cloned into the vector pKW08-Lx [39] (Addgene) in place of the *Lx* gene with using BamHI and HindIII restriction sites. Successful cloning was verified with sequencing of the appropriate region of the plasmid. 0.5–0.5 μg of pKW08-Stl and pKW08-Lx (used as control) were electroporated into electrocompetent wt *M. smegmatis* strain. Three parallel strains from each were chosen and used in the experiments. Primers used for cloning are compiled in Table S1.

2.8. Verification of protein expression by Western blot

Stl expressing *M. smegmatis* strains were grown until the OD_{600} reached 0.4–0.5 then the cells were harvested by centrifugation. Pellets were resuspended in lysis buffer (50 mM Tris–HCl, pH = 7.5; 140 mM NaCl; 1 mMEDTA; 0.5% SDS; 1% Triton X-100; 0.5 mM PMSF; 2 mMBA; 15 mM 2-mercaptoethanol; 0.1 mg/mL DNase) and sonicated (Elma, S30H ElmaSonic, D78224) for 4 times 5 min. Concentrations of the final supernatants of the cell extraction were measured using Nanodrop ND-1000 and equalized by dilution before Western-blot analysis. Protein lysates were heated at 95 °C for 5 min, separated by SDS-PAGE, and transferred to PVDF membrane for immunoblotting with the specific antibody against AU1 epitope-tag (Novus Biologicals). Immune-complexes were visualized using enhanced chemiluminescence. The blotted polyacrylamide gel stained with Comassie-Brilliant Blue and the PVDF membrane stained with Ponceau were used as loading controls.

2.9. dNTP extraction

Exponential phase cells were grown with appropriate antibiotics until OD_{600} = 0.6. The total CFUs were determined for each culture, and cells were centrifuged for extraction. Washed pellet were extracted in 0.5 mL ice-cold 60% methanol overnigth at −20 °C. Cells were removed by centrifugation (15–20 min, 13,000 rpm) the methanolic supernatant was boiled for 5 min and centrifuged. Soluble dNTP fraction containing supernatant were evaporated under vacuum (Eppendorf) at 45 °C, 1 h. Extracted dNTPs were dissolved in 50 μL dUTPase puffer (30 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl, 1 mM EDTA) and stored at −80 °C.

2.10. Determination of the pyrimidine pool size

Determination of the dCTP and dTTP pool size in each extract was based on DNA polymerase-catalyzed incorporation of radioactive dNTP into synthetic oligonucleotide templates [40]. The reaction mixture (50 μL) contained *Klenow* buffer, 0.5 unit exonuclease negative *Klenow*-fragment (Fermentas), 0.25 μM dTTP/dCTP specific template, 0.25 μM primer (Table S1), 8 μL dNTP-extract 2.5 μM [3H] dATP (1,5 Ci/mmol) (American Radiolabeled Chemicals Inc.). Incubation was carried out for 60 min at 37 °C and reaction mix was spotted onto DE81 paper. The papers were dried, washed (3 × 10 min) with 5% Na_2HPO_4, and rinsed once with distilled water and once with 95% ethanol. After drying, radioactivity on the papers was measured in a liquid scintillation counter (Beckman). In case of dCTP measurement we used Taq polymerase (RedTaq, Sigma) as Klenow polymerase is capable of incorporating CTP and GTP from nucleotide extracts [41]. Incubation was carried out at 48 °C for 1 h. Two-fold dilution series were prepared from cell extracts in the PCR reactions. Three parallel strain were used from each mutation in the experiments.

2.11. Determination of the dUTP pool size

dUTP concentration was measured according to Koehler and Ladner [42]. The samples used for dTTP measurements were distributed into two equal portions. The first portion was treated with 40 ng recombinant dUTPase ($mtDUT^{H145W}$) [20] at 37 °C, 45 min, then dUTPase enzyme was precipitated with 60% methanol. Therefore, in the first portion, dUTP was hydrolyzed while in the second portion (not treated with dUTPase) both dTTP and dUTP remained present. The dUTP and dTTP levels were calculated as follows: $dUTP = dTTP_{non\text{-}treated} - dTTP_{treated}$; $dTTP = dTTP_{treated}$.

2.12. Genomic DNA isolation

10 mL liquid culture was grown until OD_{600} of 0.5 and harvested. The cells were resuspended in 1 mL 10 mM Tris, pH 7.5 and 0.1 mm glass beads were added to 2 mL volume. The cells were disrupted by vortex and ice incubation by turn. After centrifugation the supernatant was manipulated routinely to purify DNA by phenol:chloroform:IAA (25:24:1) extraction followed by isopropanol precipitation.

2.13. Determination of genomic uracil content

In order to quantify uracil content of DNA, a real-time quantitative PCR-based assay was used [43]. Genomic DNA was isolated and digested with BamHI. DNA fragments of 5 kb were purified from gel for enrichment of specific fragments. Real-time PCR was performed on Mx3000P qPCR System (Agilent Technologies) using EvaGreen dye (Biotium) and PfuTurbo Hotstart DNA polymerase (Stratagene) or Mytaq Hotstart DNA polymerase (Bioline). A segment with 1017 base length defined by the primers (Table S1) is amplified during the PCR reaction. As described previously, the detection limit of this method is around 100 uracil/million bases when the PCR template used is of approx. 1000 bases [43]. Two-fold dilution series were prepared from DNA samples. Three parallel strain were used from each mutation in the experiments.

2.14. Growth inhibition test

M. smegmatis strains were growed in liquid media until saturation and induced with 20 ng/mL tetracyclin. 10-fold dilution series were plated after 0, 1, 2, 4, 8 and 24 h of induction. CFU was counted

and normalized to $t = 0$. Three parallel strains and controls were used in the experiments.

3. Results

3.1. Stl binds to M. tuberculosis dUTPase in vitro

Previous studies showed that Stl is capable to form a complex with *S. aureus* phage *Φ11* dUTPase [33]. To investigate whether Stl may also bind to mycobacterial dUTPase (mtDUT), we carried out native gel electrophoresis experiments. As shown in Fig. 1A, when the mixture of the two proteins is run on the gel, we can only observe a very slight, if any, band corresponding to the positions of the free proteins, however, a new band appears putatively corresponding to the complex of these two proteins. These results clearly suggest that mtDUT and Stl form a stable complex. Complexation is most evident at 1:1 ratio of Stl and mtDUT subunits.

3.2. Stl inhibits the enzymatic activity of M. tuberculosis dUTPase in vitro

We have previously found that Stl inhibits the enzymatic acitivy of *S. aureus* phage *Φ11* dUTPase [33]. The results suggested a slow and tight binding interaction between Stl and dUTPase. It was also shown that Stl and dUTP compete for the active sites of dUTPase. We designed *in vitro* experiments with mtDUT to investigate whether Stl can also inhibit this mycobacterial enzyme. We tested the Stl inhibition on the wild type enzyme ($mtDUT^{WT}$) and on the quasi wild type variant ($mtDUT^{H145W}$) containing a tryptophan substitution at the active site [20,44]. The active site architecture of dUTPase is well-known from the literature [45–48]. The mutated position is conserved for aromatic residues. The conserved Phe/Tyr/Trp at this position stacks over the uracil ring of the substrate that contributes to the catalytic efficiency of dUTPase reaction [44]. Aromatic substitution at this position does not alter the mechanism of action [49].

In both cases, a very strong inhibition of dUTPase enzymatic activity exerted by Stl was observed (Fig. 1B). Both k_{cat} and K_M values were changed as a result of Stl inhibition, indicating a mixed kinetic mechanism of inhibition that may suggest allosteric behavior. However, further kinetic experiments are required for an in-depth characterization of the mechanism of inhibition, these will form the basis of a further study. Similarly to the measurements with *Φ11* dUTPase, Stl inhibition is only observed in case the dUTPase variants are pre-incubated with Stl before dUTP addition. This indicates that the formation of the mtDUT:Stl complex is slow as compared to substrate binding (*cf.* [33]). Results of a titration experiment with varying Stl concentration indicated that the inhibitory effect of Stl is associated with a very low apparent inhibitory constant (K_i in the nanomolar range, Fig. 1B, Table 1). This K_i value is on the same low range as found in the case of the Stl-induced inhibition experiments of the *S. aureus* phage *Φ11* dUTPase [33], suggesting that the complexation of Stl with the two dUTPases are similarly strong. However, the maximal inhibition is about 80%, which value is somewhat lower as compared to the *Φ11* dUTPase (100%) (Fig. 1B, Table 1).

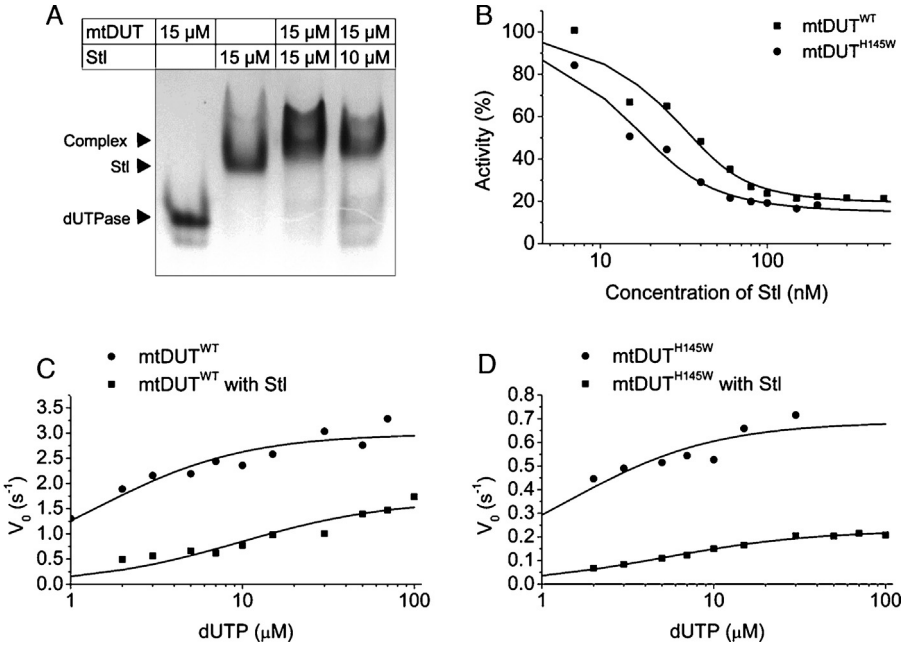

Fig. 1. Stl forms a stable complex and inhibits *Mycobacterium tuberculosis* dUTPase *in vitro*. (A) Native gel electrophoresis experiment indicates stable complex formation between Stl and MtdUTPase. Species and concentrations given in monomers are indicated. (B) Representative measurements of Stl's 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 (Eq. (1)) to yield apparent $K_i = 5.5 \pm 4.6$ nM for $mtDUT^{WT}$ and apparent $K_i = 4.4 \pm 2.8$ nM for $mtDUT^{H145W}$. The total amplitudes of the activity change were $83.8 \pm 6.7\%$ for $mtDUT^{WT}$ and $87.1 \pm 5.8\%$ for $mtDUT^{H145W}$, respectively. Figure shows a representative measurement and its analysis. Errors shown are the errors calculated in the fitting process. Each measurement was repeated multiple times. The averages and the SD-s of these measurements are summarized in Table 1. (C) Representative measurements of substrate dependence of $mtDUT^{WT}$ activity in the presence and absence of 150 nM Stl. The concentration of dUTPase was 50 nM in the measurements. Smooth lines represent the fit of Michaelis–Menten equation to the data, yielding $K_M = 1.4 \pm 0.3$ μM and $k_{cat} = 3 \pm 0.1$ 1/s in the absence of Stl, $K_M = 9.7 \pm 2.5$ μM and $k_{cat} = 1.7 \pm 0.1$ 1/s in the presence of 150 nM Stl. Figure shows a representative measurement and its analysis. Errors shown are the errors calculated in the fitting process. Each measurement was repeated multiple times. The averages and the standard deviations of these measurements are summarized in Table 1. (D) Representative measurements of substrate dependence of $mtDUT^{H145W}$ activity in the presence and absence of 150 nM Stl. The concentration of dUTPase was 50 nM in the measurements. Smooth lines represent the fit of Michaelis–Menten equation to the data, yielding $K_M = 1.3 \pm 0.4$ μM and $k_{cat} = 0.7 \pm 0.0$ in the absence of Stl, $K_M = 5.3 \pm 0.4$ $mtDUT^{H145W}$ and $k_{cat} = 0.2 \pm 0.0$ in the presence of 150 nM Stl. Figure shows a representative measurement and its analysis. Errors shown are the errors calculated in the fitting process. Each measurement was repeated multiple times. The averages and the standard deviations of these measurements are summarized in Table 1.

Table 1
Kinetic parameters of dUTPase inhibiton by Stl.

Michaelis–Menten parameters	K_M (μM)	k_{cat} (s^{-1})	Number of repeats
$mtDUT^{WT}$	1.3 ± 0.1	2.6 ± 0.6	2
$mtDUT^{WT}$ with Stl	9.4 ± 4.1	1.3 ± 0.3	4
$mtDUT^{H145W}$	1.6 ± 1.2	0.8 ± 0.2	3
$mtDUT^{H145W}$ with Stl	5.7 ± 0.9	0.3 ± 0.0	3

Stl inhibition	K_i (nM)	Inhibition (%)	
$mtDUT^{WT}$	6.8 ± 4.4	81.1 ± 10.2	3
$mtDUT^{H145W}$	4.2 ± 0.8	85.2 ± 1.5	3

Protein concentrations were as follows: $mtDUT^{WT}$ or $mtDUT^{H145W}$: 50 nM, Stl: 150 nM. Further increase of Stl concentration did not cause any additional changes. Data represent average and standard deviations of parallel measurements.

We also tested the substrate concentration dependence of dUTPase activity in the presence and absence of Stl. These results show that the maximal activity of both mycobacterial dUTPases (wild type and Trp mutant) decreases in the presence of Stl with practically same characteristics, while the Michaelis constant (K_M) increases (Fig. 1C, Table 1.). These parametric changes, together with the low K_i for Stl:mtDUT compared to the K_M for mtDUT:dUTP and the relevant observation of the requirement for pre-incubation suggest a slow and tight binding inhibition mechanism, as it was observed for *Φ11* dUTPase [33]. However, the lower value of the maximal inhibition (80% *versus* 100%) may indicate a difference in the nature of the interaction between the substrate and the dUTPase:Stl complex for mycobacterial and *Φ11* dUTPases – this difference will be investigated in forthcoming studies.

Our results also show that the tryptophan substitution at the active site in $mtDUT^{H145W}$ does not change the interaction of Stl with dUTPase. This finding will facilitate more detailed biochemical characterization of the mtDUT:Stl interaction with the help of the intrinsic tryptophan label in later studies.

3.3. Stl expression causes increased dUTP level in M. smegmatis

To investigate whether Stl can inhibit the dUTPase function in the *Mycobacterium* cell, we constructed *M. smegmatis* strains that expressed the Stl protein from the pGem-int-Stl plasmid integrated to the *M. smegmatis* genome. The expression of the AU-1 tagged Stl protein in the transformed strains was verified with Western-blotting (Fig. S1). We have determined pyrimidine nucleotide levels in cell extracts by a PCR-based method [40] and found that the levels of dTTP and dCTP were normal. However, the level of dUTP was significantly increased in the Stl expressing strains compared to control strains transformed with an empty vector (Fig. 2A). Potential increase in the genomic uracil content in the Stl expressing strains remained below the detection limit.

3.4. Stl expression perturbs colony formation in M. smegmatis

We also constructed a strain in which we could express the Stl protein in a tetracyclin-inducible manner to exclude any compensatory effect for growth. We constructed this strain by electroporating electrocompetent wild type *M. smegmatis* cells with pKW08-Stl. The tetracyclin-inducible expression of AU-1 tagged Stl protein in the transformed strains was verified with Western-blotting (Fig. 3A). Stl expression was observed after 1 h of induction and was clearly visible after 8 h of expression upon the addition of 20 ng/mL tetracyclin (Fig. 3A). Without tetracyclin induction, the basal transcription level seemed to be lower than that in the 1-h post-induction sample. We found that upon induction of Stl expression, the ability of colony formation was drastically decreased (the number of colony-forming units (CFU) remained

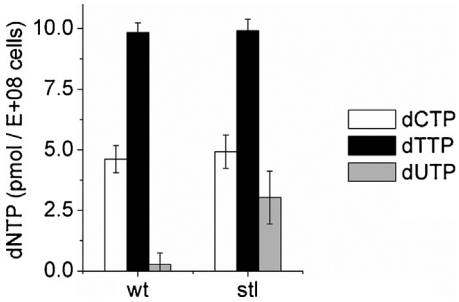

Fig. 2. Stl expression leads to an increased dUTP level in *Mycobacterium smegmatis*. Pyrimidine nucleotide pool data measured in *Mycobacteria* either expressing or not expressing Stl protein. dTTP, dCTP and dUTP were measured with a PCR-based method. dUTPase treatment allows to differentiate between 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.

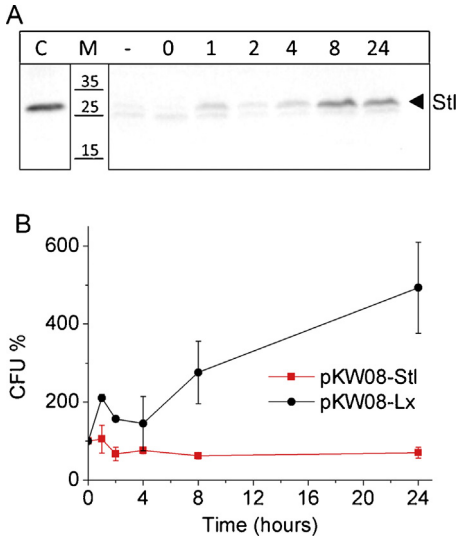

Fig. 3. Stl expression interferes with colony formation in *Mycobacterium smegmatis*. (A) Expression of AU-1 tagged Stl after induction with tetracyclin in different time points (0, 1, 2, 4, 8 and 24 h after induction). 'C' indicates constant Stl expression from pGem-int-Stl vector, 'M' indicates protein marker and '–' indicates negative control. (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.

constant) as compared to the control strains in which luciferase (Lx) expression was induced (transformed with pKW08-Lx) [39] (Fig. 3B). Consistently, we also found much less colonies after the electroporation of 0.5 μg pGem-int-Stl into electrocompetent *M. smegmatis* as compared to the control case (data not shown).

4. Discussion

The uracil-DNA glycosylase inhibitor (*Ugi*) protein, isolated from the *B. subtilis* bacteriophage *PBS2*, is a well known and generally used inhibitor of the Ung enzyme from various species [28,29,50,51]. Although the presence of potential dUTPase inhibitor proteins was also suggested in bacteriophage *PBS2* infected *B. subtilis* cells as well as in *D. melanogaster* cell extracts [30,31], till now none of these suggestions could be confirmed at the molecular level. Here we show that Stl, a repressor protein of *S. aureus* is not only a potent inhibitor of the *Staphylococcal Φ11* phage dUTPase [33], but also exerts cross-species effects by inhibiting mycobacterial dUTPases both *in vitro* and in the bacterial cell.

Results presented in Fig. 1 show that Stl and *M. 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 inhibitor protein led to high cellular dUTP levels and moderate colony forming in *M. smegmatis* cells (Figs. 2 and 3). However, Stl expression did not perturb the cellular levels of other pyrimidine nucleotides (*i.e.* dCTP and dTTP). In a recent study, we showed that a mycobacterium specific structural motif is essential for viability potentially due to a yet unidentified protein-protein interaction [23]. This putative interaction was most likely not perturbed by Stl, as the bacteria expressing the exogenous inhibitor protein remained viable, although colony formation was decreased. We also measured the genomic uracil content of the Stl expressing strains but could not detect significant increase. Hence, most of the dUMP moieties incorporated into DNA under the high cellular dUTP concentration environment could have been repaired by the uracil excision repair enzymes. In addition to the usually present uracil-DNA glycosylase enzymes, *Mycobacteria* encode an additional uracil DNA glycosylase (UdgB) [52,53], potentially facilitating efficient repair.

Although the lack of conventional mismatch repair (MMR) and the presence of error prone polymerases present clear variableness which may denote evolutionary advantage for *Mycobacteria* [8], our result on colony forming ability upon Stl expression indicates that the preservation of the normal nucleotide pool and genomic integrity is of high importance in *Mycobacteria*. The presence of multiple enzymes with similar functions in preventive DNA repair mechanisms and in the base excision repair (*i.e.* two dUTPases (dUTPase and a bifunctional dCTP deaminase/dUTPase) and two uracil DNA glycosylase enzymes) also indicates the importance of these pathways. According to our *in vitro* results, we expect about 20% of the dUTPase activity remaining in the Stl-expressing mycobacterial cell and still, we detected a clear phenotype (*i.e.* increasing dUTP level and decreased colony formation) in contrast with almost complete inhibition of dUTPase by siRNA in human cells where such inhibition had no detectable effect on the cellular phenotype only in the additional presence of FdUrd [54,55].

According to our results we propose Stl as a reagent to be used in dUTPase inhibition experiments either *in vitro* or *in situ*/*in vivo* in a manner analogous to experiments using Ugi as the inhibitor of Ung from various species. Several small molecule inhibitors against human dUTPase were recently published [56–59]. We, however, propose that proteinaceous inhibition of dUTPase is also possible and produces a clear cellular phenotype in *Mycobacterium*. The use of the exogenously expressed Stl protein in *Staphylococcus* and *Mycobacteria* is expected to become an effective tool in studying nucleotide metabolism pathways within the cellular environment. It remains to be seen if Stl may also inhibit eukaryotic dUTPases, as well.

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Authors contribution

Conceived and designed experiments: RH, JESZ, KNY, SZT, PD, JT and BGV. Performed experiments: RH, JESZ, SZT, PD and KNY. Analyzed data: RH, JESZ, SZT, PD and KNY. Wrote paper: RH, JESZ, KNY, SZT, PD, JT and BGV.

Conflict of interest statement

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.dnarep.2015.03.005.

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