**Ph.D. Thesis summary**

# An IS element-mediated synthetic biology approach for engineering multi-copy integration of large genetic constructs into bacterial genomes

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## Introduction

*E. coli* has long been used for the expression of foreign proteins and for the production of valuable metabolites by expression of complete metabolic pathways. Using episomal (plasmid) DNA is convenient but has drawbacks including increased metabolic burden, the requirement for selection in the form of antibiotics, instability and gene copy number variation. Chromosomal integration offers a stable and selection-free alternative to using DNA plasmids for expression of foreign proteins and metabolic pathways.

Small fragments of DNA could be inserted into bacterial genome by homologous recombination but integration of larger DNA needs endonuclease assisted recombineering. Both kinds of methods are tedious and results into single copy integration. Multi-copy transgene integration needs landing pads to be inserted in genome prior to transgene integration which is tedious and time taking. Tn7 mediated method is based on site specific recombination but for multi-copy integration of transgene we need to insert recombination sites (landing pads) in genome first. Requirement of landing pads is usually a limiting factor for chromosomal integration so we introduce the use of IS (Insertion sequences) elements as natural landing pads. Insertion sequence (IS) elements are segments of bacterial DNA that can move from one position on a chromosome to different position on the same chromosome or on a different chromosome. IS elements are not essential for the survival of an organism, are available in multiple copies in the genome and are ubiquitous (present in multiple species), which simplifies genome editing in multiple organisms. Furthermore, they may allow the amplification of transgenes in a second step. CRISPR/Cas9 has been demonstrated as a robust genome engineering tool for eukaryotic as well as prokaryotic organisms. Here we developed a CRISPR/Cas9 assisted method for multi-copy integration of transgene at IS element in bacterial genome followed by copy number amplification of already inserted DNA.

## Aims

We aimed to develop a natural landing pad-mediated synthetic biology toolkit that allows integration and amplification of heterologous DNA into the bacterial genome. We wished to answer the following questions:

1. Can we develop a system to clone foreign DNA in a bacterial

genome in multiple copies using insertion sequences as targets?

2. Can we further increase the copy number of the inserted DNA by replicative transposition?
3. How does the stability of the multi-copy genomic construct compare to that of the plasmid-based construct?

## Methods

### 1. Integration of resistance genes into genomic IS elements by recombineering

We expressed λ-Red recombineering enzymes in target bacteria followed by transformation of template cassette. A fraction of recovery culture was spread on the respective antibiotic plate. Recombinants were screened by colony PCR where one primer was binding on template and other on neighboring region on bacterial genome.

### 2. Integration of a resistance gene or the *vioABCDE* operon into genomic IS elements using NO-SCAR

vio*ABCDE* operon as well as resistance gene were inserted in to two copies of respective IS by NO-SCAR system where CRSIPR/Cas9 was applied along with recombineering.

### 3. Integration of non-selectable genes into genomic IS elements using NO-SCAR

Non-selectable gene (gfp) was inserted in to single copy of respective IS by NO-SCAR system where CRSIPR/Cas9 was applied along with recombineering.

### 4. Integration of marked IS elements into the genome using transposition

IS3::SpR cargo was amplified from MDS30::SpR genome following by cloning into pSG78A plasmid which is a conditional (needs π protein) replicative plasmid. This donor plasmid was transformed into ISs free host having IS3 transposase expressing plasmid followed by spread of a fraction on Sp plate. One day later, IS*3*::SpR were localized into $Sp^R Cm^S Ap^S$ colonies (i.e., those

that have lost both plasmids but retain the IS*3*::SpR) using ST-PCR.

## 5. Semi-random two-step PCR (ST-PCR)

Genomic DNA was PCR-amplified from the colonies of interest first using primer pairs Sp439Rev + CEKG2B and Sp110Fwd + CEKG2B. The DNA product generated by the first PCR was diluted 5x with TE buffer (10 mM Tris, 1 mM EDTA) and subsequently used as a template for nested PCR, with primer pairs SmFwd + CEKG4 and Sp347Fwd + CEKG4 corresponding to the first two PCRs, respectively. PCR products were sequenced with SmFwd or Sp347Fwd (depending on which was used for the nested PCR).

## 6. Amplification of IS elements marked with antibiotic resistance genes

Bacterial strains already having antibiotic resistance gene at target IS element, were transformed with respective transposase expressing plasmid. Transposase gene was expressed by adding aTC inducer and a small fraction was spread on respective antibiotic gradient plate. Few colonies from high edge were screened by ddPCR to detect the transgene copy number.

## 7. Quantification of violacein production

To quantify the violacein production of liquid *E. coli* cultures, we applied the modified protocol of Zhu et al. (Zhu et al., 2011) which is an DMSO based method.

## 8. Monitoring the stability of violacein production

The stability of violacein production was assessed in two different experiments. The first type of experiment monitored the ratio of purple colonies in the lack of antibiotic selection. The second type of experiment monitored the violacein production of liquid cultures grown in the lack of selection.

## 9. Droplet digital PCR

To determine the copy numbers of marked IS elements within the bacterial chromosome, droplet digital PCR (ddPCR) experiments were performed using the EvaGreen protocol of BioRad QX200 Droplet DigitalPCR system (BioRad,

Hercules, CA, USA).

### 10. Whole genome sequencing of bacteria

For whole-genome shotgun sequencing, a genomic library was prepared from four strains (B0, B1, B2, and B3) using the Nextera XT Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol. The libraries were sequenced with Illumina NextSeq 500 sequencers using 2×150 PE sequencing.

## Results

### 1. Recombineering-mediated integration of violacein operon was achieved in single copies using antibiotic selection

Since the integration of the violacein operon into the genome of *E. coli* was not possible using visual screening, we inserted an antibiotic resistance gene (SpR or KmR) inside the cassette next to the *vio* operon, within the segment flanked by IS-specific homologies. It was done by pORTMAGE4 mediated recombineering. Four such plasmids were generated: pST76AIS1X::*vioABCDE*_SpR, pST76AIS1X::*vioABCDE*_KmR, pST76AIS3X::*vioABCDE*_SpR and pST76AIS3X::*vioABCDE*_KmR. Again, linear DNA cassettes for recombineering were generated from these plasmids by restriction digestion. When electroporating these cassettes, we obtained mixed results: on the one hand, if targeting genomic IS*1* in MDS42IS*1*, correct recombination events could be verified, but none of the co-integrant colonies were purple. This was the case even though all four plasmids used to generate the cassettes granted their host a dark purple colony phenotype. On the other hand, when targeting the IS*3* of MDS30, the PCR-verified genomic co-integrants displayed a purple color: Three out of nine colonies (33%) were PCR-positive when using SpR, and 9/10 (90%) were positive when using KmR as a selection marker. Double co-integrants were never obtained this way. We have nevertheless demonstrated that the 9074 bp and 9359 bp-long *vioABCDE*-SpR and *vioABCDE*-KmR cassettes, respectively, could be targeted into genomic IS*3* elements by recombineering. In both experiments targeting IS*3* of BLK09 or BLK16, the majority (>90%) of the colonies displayed a purple color, indicating

a relatively low rate of false-positive resistance. In strain MDS27 however, <50% of colonies were purple to the naked eye, and some of the white colonies were true co-integrants verified by PCR. This exemplifies the strain dependence of the ratio of colonies with the functional expression of the inserted operon. The bias of the integration seen for resistance genes was also present when inserting the five-gene operon, although to a variable extent.

## 2. CRISPR/Cas9-assisted recombineering allowed the genomic integration of an unselectable gene in a single copy

Besides generating double co-integrants, the great value of applying CRISPR/Cas9-based facilitation was unveiled upon the integration of unselectable genes into the chromosome. Peculiarly, we managed to insert the *gfp* gene into the IS*3* of *E. coli* MDS30 using recombineering and subsequent CRISPR/Cas9 selection. In the course of these experiments, we demonstrated that a 20 nt spacer is equal or superior to a 30 nt spacer in pCas9IS*3* concerning efficiency, and up to 20% (2/10) of colonies obtained proved to be positive by PCR. The integration of *gfp* into the IS*1* element of MDS42IS*1* was successfully achieved using recombineering and concomitant CRISPR/Cas9 cleavage provided by the NO-SCAR system (plasmids pKDsg-IS1 and pCas9cr4). In the latter case, 3 out of 30 colonies (10%) were PCR-positive. The elevated green fluorescence levels of both engineered strains were verified using a microplate reader.

## 3. Genomic integration of a five-gene operon in single or double copies using CRISPR/Cas9-assisted recombineering

Next, we tested the effect of CRISPR/Cas9 cleavage on the integration efficiency of long (>9 kbp) selectable DNA cassettes. Since integrating our Vio operon into IS*1* targets by recombineering led to the loss of violacein production (see above), we focused on experiments targeting IS*3*. First, we tested the effect of subsequent Cas cleavage using pCas9IS3. We found that in most of the experiments targeting MDS27, BLK09, or BLK16 (using either SpR or KmR as selection markers), subsequent Cas cleavage not only failed to improve the efficiency of pORTMAGE-mediated recombineering but on many occasions the recombinant cells were missing altogether. In a few cases nevertheless, we did manage to obtain single *vioABCDE*_KmR insertions into either of the IS*3* elements of BLK09 or BLK16 but double co-integrants were not obtained this

way. Second, we tested the effect of concomitant Cas cleavage using the NO-SCAR system. We started by inserting the *vioABCDE*_KmR cassette into the single IS*3* of MDS30 using NO-SCAR (plasmids pKDsg-IS3 and pCas9cr4). We obtained single co-integrants with low absolute efficiency (on the order of $10^{-2}$ correct colonies/ng), the high ratio of correct colonies (6/10) nevertheless permitted the easy detection of true recombinants. The same system was used to target the two copies of IS*3* residing in MDS27 as well as two copies of IS*1* residing in MDS39R2. We observed 7.7% of the colonies in MDS27 and 5% of the colonies in MDS39R2 to harbor the operon at both loci.

## 4. Copy number amplification of cargo genes, using copy-paste transposition of IS elements, was achieved with the thermo-sensitive version of transposase-expressing plasmids

To allow the easy elimination of high-resistance cells resulting from transposition of the marked IS into plasmids, we engineered temperature-sensitive transposase plasmids (pSTinsAB'tetR and pSTtnp3tetR for IS*1* and IS*3*, respectively). We also modified the copy-amplification protocol so that after transposase expression, the cells are plated on gradient plates only after the plasmids had been cured from the cells (as described in the Methods section). This guaranteed that only chromosomal co-integrants of the resistance gene are selected after transposase expression.
Due to the low reproducibility of IS*1*-mediated amplification and to the fact that violacein expression was not observable upon IS*1*-targeted integration (described in the previous section), we focused primarily on amplification of the violacein operon (*vioABCDE*) using IS*3* elements. Strains of *E. coli* BLK16_IS*3*::*vioABCDE*_SpR, generated by recombineering (see above) were used as starting points. These carried a single copy of the violacein operon inside the IS*3* element residing at locus 1, along with an SpR marker. After the first round of transposase expression, we analyzed five colonies by ddPCR (targeting the resistance marker) from the high-end of the gradient plates and found the mean copy number to be 5.50 (±1.22). Choosing a colony displaying 7.59 copies and re-expressing the IS*3* transposase led to a further significant elevation of the mean copy number within colonies displaying high resistance levels (8.03±1.21, n=4). Again, we chose the colony displaying the highest copy number (9.10) and expressed the IS*3* transposase for the third time. The mean copy number of the colonies analyzed in the third round did not significantly differ from those of the second round. However, by 'cherry picking', we were still able to find individual

clones displaying further elevated copy numbers.

## 5. Whole-genome sequencing confirmed the multiple copies of the *vioABCDE* operon in the genome of engineered strains

Next, we sent a series of four strains obtained from the copy-amplification experiment described above (the starting strain and one clone from each round of transposase induction) for whole-genome sequencing. Our aims were i) to verify the copy numbers of the amplified IS*3*::*vioABCDE* _SpR elements, ii) to analyze the fraction of mutant or truncated *vioABCDE* operons, iii) to test whether amplification leads to tandem repeats or a random scatter of the loaded IS*3* element within the genome and iv) to check for unexpected genomic rearrangements. The sequence of the starting strain (B0) confirmed the presence of the *vioABCDE* operon, along with the SpR marker within the IRs of the IS*3* element residing at locus 1 (genomic coordinates 649,839-659,074). The sequence of strain B1, obtained in the first round of transposase induction displayed a 5.71-fold elevated relative coverage of the loaded IS*3*, and unexpectedly, of a 14 kbp segment directly downstream of the IS (amplified region: 649,839-673,907). This stands in good agreement with the 5.59 copy numbers measured by ddPCR. Sequencing of strain B2, which is not a direct descendant of B1, revealed an 11.32-fold increased relative coverage of a similarly large genomic segment delimited on the left side by the left IR of the loaded IS*3* (coordinates: 649839-677086). Interestingly, it also displayed a further 5.44 copies (totaling 16.76) of an anterior segment (659074-667565), which is directly downstream of the manipulated IS. The approx. 11-fold increase in the copy number of the violacein operon roughly supports the 9.1 copies indicated by the ddPCR. Finally, strain B3, a descendant of B2, was expected to harbor 26.11 copies of the resistance marker, based on ddPCR. Sequencing did not confirm this, the relative coverage of the genomic region encompassing the loaded IS*3* (649839-677086) nevertheless increased from 11.32 to 12.51, and the number of additional copies of the interior segment downstream of the IS (659074-667565) decreased from 5.44 to 3.54. We, therefore, concluded that although the third round of induction did not increase the mean copy number of the inserted operon, screening a low number of colonies (<10) permitted the identification of a clone that displayed further amplification of the transgenes. We note that in another clone of the same series of induced strains we detected 9.89 copies, and in another induction series done in parallel, we measured 10.71 and 11.83 copies of SpR using ddPCR. We also

experienced that in a small fraction of analyses (1-5%), the ddPCR results were misleading (data not shown), underlining the importance of result verification. The sequenced strains did not harbor the loaded IS*3* scattered throughout the genome, but rather amplified in tandem repeats. However, the IS copies did not form a back-to-back array since, rather unexpectedly, they were amplified together with a large (14-18 kbp) DNA segment lying directly downstream to them.

### 6. Violacein production was proportional to the copy number of the operon encoding its production

Violacein content of the starting strain and its derivatives obtained after various rounds of transposase induction were routinely measured. The amount of violacein released from the BLK16IS*3*::*vioABCDE*_SpR derivatives displayed a good correlation ($R^2$=0.98) with the copy number of the *vioABCDE* operon inferred from its relative sequencing coverage.

### 7. Phenotypic stability of genomic co-integrants was superior to their plasmid-based counter-parts under selection-free conditions

A key phenotypic trait of engineered strains is the stability of expressing the inserted transgenes in the lack of selection. We compared strains harboring chromosomal *vioABCDE* operons to those carrying the same operon on multi-copy plasmids using two different tests. Our first test monitored the fraction of cells displaying violacein production. We chose three well-known strains, *E. coli* BL21(DE3), DH5α, and MG1655 to be our controls, all transformed with the pUTLIQ_*vioABCDE* plasmid. From our collection of genome-engineered strains we tested three versions of BLK16IS*3*::*vioABCDE*_SpR (carrying 1, 5.59 or 10.5 copies of IS*3*::*vioABCDE*_SpR, respectively), and strain MDS27IS*3*::*vioABCDE*_KmR, carrying 5.8 copies, according to our ddPCR measurements. All three control strains displayed a steep decrease in the ratio of purple colonies and completely lost the purple phenotype by generation 40. On the contrary, all four genomic co-integrants tested in this experiment retained purple color in >96% of their colonies, with the single copy strain not displaying loss of function at all. We note that i) multi-deletion strains (MDS42 and BLK16) of *E. coli* displayed complete plasmid loss even faster than their conventional, non-reduced counterparts, and ii) further single and the double copy-harboring strains [BLK16IS*3*::*vioABCDE*_SpR (1 copy, locus 2) and

MDS27IS*3*:: *vioABCDE* _KmR, 2 copies, respectively] did not display any loss of function either. We, therefore, conclude that our genomic co-integrants can be used for the prolonged expression of transgene arrays in the lack of selection with a negligible fraction of cells displaying a complete loss of production. Our second test assaying the stability of violacein production in the lack of selection measured the levels of violacein extracted from the cultures each day. As expected, we saw the rapid decline of violacein levels in the case of *E. coli* BL21(DE3), MG1655, and MDS42 strains initially harboring the pUTLIQ_*vioABCDE* plasmid. *E. coli* DH5 $\alpha$ displayed a somewhat smaller, but still significant loss of 35% ($P=0.012$ with a two-tailed, unpaired t-test). In contrast, no significant decrease in the violacein levels was observed on day 4 (compared to day 1) for strains BLK16IS*3*::*vioABCDE*_SpR carrying 5.59, 9.1, and 10.5 copies of the violacein operon, respectively, and strains MDS27IS*3*::*vioABCDE*_KmR carrying 2 and 5.8 copies, respectively. Furthermore, in this test, the violacein levels produced by MDS27IS*3*::*vioABCDE*_KmR carrying 5.8 copies significantly surpassed that of all other strains, including the four strains carrying the pUTLIQ_*vioABCDE* plasmid.

## 8. Transposition of marked IS*3* elements into the *E. coli* chromosome

Finally, we asked whether IS*3* can be transferred into a bacterial strain of choice by controlled transposition. This process, which can be referred to as a potential “Step 0” of the inPOSE protocol could be valuable for future users who wish to utilize IS*3* for chromosomal cloning and amplification inside a strain that has no copies of this element at all. For the process of IS*3* entry into the chromosome, we decided to use transposition, catalyzed by the plasmid-encoded transposase used in the transgene copy-amplification experiments above. To make the IS*3* entry selectable, a marked version of IS*3* was required, we, therefore, used the IS*3*::SpR allele generated in the course of SpR recombineering in MDS30 (see the section on "Targeting ISes by recombineering" above). The IS*3*::SpR cassette was PCR-amplified from the chromosome and cloned into a suicide plasmid that is unable to replicate in strains lacking the *pir* gene. This donor plasmid, pSG78A_full_IS*3*::SpR was transformed into *E. coli* MDS42 carrying the transposase-expressing pSTtnp3tetR plasmid, followed by plating on Sp plates. Replica plating revealed that 10% (2/20) of the obtained colonies were $Sp^RCm^SAp^S$, indicating that they had lost both the donor plasmid and the transposase plasmid, but retained the SpR gene, presumably due to its transposition into the genome. To verify transposition and identify the locus of

entry, we carried out ST-PCR on the chromosomal DNA prepared from two colonies. Two pairs of PCR reactions were carried out, as described in the Methods. Sequencing a unique product (from SmFwd + CEKG4 PCR on colony 11) confirmed IS*3*::SpR insertion into the *pqiA* gene at position 1,012,519 (using NC000913.3 coordinates). In a similar experiment, the transposition of IS*3*::SpR into MDS30 was induced and selected, and this time, 33% (10/30) of the colonies displayed a Sp$^{R}$Cm$^{S}$Ap$^{S}$ phenotype. Most of the ST-PCR reactions carried out on the genomic DNA of ten such colonies generated unique PCR patterns, probably indicating different points of insertion. Sequencing a PCR product from one of the least complex patterns (Sp347Fwd + CEKG4 PCR product of colony 8) revealed IS*3*::SpR integration into the 5' untranslated region of the *waaL* gene (position 3,796,945, NC000913.3 coordinates). We, therefore, demonstrated the transposition-mediated entry of IS*3*::SpR into two different strains of *E. coli* (one IS-free, the other harboring a single copy of IS*3*), with IS*3*::SpR entering the chromosome at two distinct loci.

## Summary

Plasmids are the most common tools used to clone and express a gene of interest in bacteria. Plasmid-based gene expression, however, has multiple drawbacks: as the bacterial cells divide there is variation in the copy number of the plasmid received by the offspring which affects the expression level of the encoded protein. The random inheritance of the plasmids also promotes the segregation of mutants and the loss of the desired function. The maintenance of plasmids always requires selection, which causes antibiotic contamination of the final product and hence puts an economic burden on the industry due to the removal step. Multi-copy integration of a transgene into the bacterial chromosome could be a viable alternative to avoid these problems. Several techniques have been developed with such aims, however, very few projects have succeeded in achieving true gene amplification. In most cases, the prior insertion of a DNA sequence (the landing pad) into the bacterial genome is needed in multiple copies to allow the multi-copy integration of the transgene, which is tedious and time-consuming.

Insertion sequences (IS elements or ISes) are the simplest kind of transposable elements found in bacterial genomes. They are not essential for the survival of the host bacteria and are usually present in multiple types and copies

within most strains of most species. Our research focused on developing novel methods for easier engineering of bacterial strains that permit stable and antibiotic-contamination-free production of bioactive compounds for industrial applications. We show that bacterial insertion sequences can be used as robust genome engineering tools for this purpose.

We first aimed to develop a system allowing the one-step integration of a marked IS*3* element into the genome of a bacterium devoid of the element, and then find the locus of integration. We constructed a conditionally replicative plasmid encoding an IS*3* element marked with a spectinomycin resistance gene (SpR), and a plasmid carrying the corresponding transposase gene in an inducible form. We demonstrated the integration of the marked IS*3* at two distinct loci in two different strains of *E. coli* using this system.

In the next step we demonstrated the integration of 1 kbp long selection markers and a 9 kbp long marked operon into IS elements residing on the bacterial genome using one-step λ-Red recombineering. To further extend the possibilities of recombineering, we combined it with concomitant or subsequent cleavage of the targeted IS elements using the CRISPR/Cas9 system. This permitted the integration of resistance genes (SpR, CmR) in two copies and of an unmarked gene (*gfp*) in a single copy into the *E. coli* chromosome. CRISPR/Cas-mediated recombineering applying concomitant cleavage was also essential to integrate two copies of the marked five-gene operon into the genome in a single round.

Our next goal was to amplify the copy number of the chromosomally inserted resistance genes or the marked *vioABCDE* operon. We developed inducible IS*1* and IS*3* transposase expressing thermosensitive plasmids for this purpose. After transposase expression, we applied selection on antibiotic gradient plates to obtain bacterial colonies having multiple copies of the heterologous cargo. We monitored the copy-amplification process with droplet-digital PCR and verified the copy numbers of the amplified IS elements using Illumina sequencing.

Finally, we set up two different experimental systems to test the stability of the engineered strains carrying the *vioABCDE* operon in single or multiple copies on their chromosomes. In all experiments carried out in the lack of selection, the stability of the genomic co-integrants surpassed that of the strains carrying the same operon on multi-copy plasmids.

Overall we have shown that a selectable gene, an unselectable gene, and a marked multigene operon can be inserted into the bacterial in one or two copies by recombineering or CRISPR/Cas9-assisted recombineering. We show that a resistance gene, as well as a marked multigene operon, can be amplified to up to 12 functional copies in the genome by expressing the corresponding transposase. We call this strategy inPOSE, referring to transgene insertion and amplification by transposition. We recommend using inPOSE for the construction of bacterial strains designed for prolonged-expression of transgenes from their chromosomes.

**Thesis points:**

1. Insertion sequence elements IS*1* and IS*3* can both be used as target sequences (landing pads) to integrate an antibiotic resistance gene alone or together with a multi-gene metabolic operon into the *E. coli* chromosome by linear DNA-mediated recombineering.
2. Cleavage of the genomic target using the CRISPR/Cas system was essential for the genomic insertion of the selectable gene or operon in two copies in a single step of recombineering.
3. Cleavage of the genomic target using the CRISPR/Cas was also essential for the insertion of a non-selectable gene (*gfp*) in a single copy using recombineering.
4. The operon inserted into the genome could be copy-amplified *in vivo* by the expression of the transposase enzyme corresponding to the targeted IS element, reaching copy numbers of >10.
5. In the lack of antibiotic selection, the function of the five-gene metabolic operon was maintained for over 40 generations by the genome-engineered cells, as opposed to cells losing the operon within 10-20 generations if encoded on a plasmid.

**List of publications**

**MTMT identification number:** 10082382

1. **Shukla, R.D**.; Zvara, Á.; Avramucz, Á.; Biketova, A.Yu.; Nyerges, A.; Puskás, L.G.; Fehér, T. inPOSE: A Flexible Toolbox for Chromosomal Cloning and Amplification of Bacterial Transgenes. Microorganisms 2022, 10, 236. https://doi.org/10.3390/microorganisms10020236 (served as the basis of the current PhD dissertation). IF: 3.84

   **# This publication served as the basis of this PhD dissertation**

2. Chauhan IS, **Shukla R,** Krishna S, Sekhri S, Kaushik U, Baby S, Pal C, Siddiqi MI, Sundar S, Singh N. Recombinant Leishmania Rab6 (rLdRab6) is recognized by sera from visceral leishmaniasis patients. Exp Parasitol. 2016 Nov; 170:135-147. https://doi.org/10.1016/j.exppara.2016.09.010. **(#equal first authors**). IF: 2.011

3. Møller-Olsen, C., Ho, S.F.S., **Shukla, R.D**. *et al.* Engineered K1F bacteriophages kill intracellular *Escherichia coli* K1 in human epithelial cells. *Sci Rep* 8, 17559 (2018). https://doi.org/10.1038/s41598-018-35859-6. IF: 4.379

**Awards/Scholarships:**

**2021:** Travel grant from Biochemical Society to attend Synthetic Biology UK conference on 22-23 November 2021 in Nottingham, UK

**2021:** Delivered a talk on my PhD project at Synthetic Biology UK conference on 23 November 2021 in Nottingham, UK

**2019:** Travel grant to attend EMBO Practical Course: Synthetic Biology in Action: Bridging Natural/Non-Natural, 15 -22 September 2019, EMBL Heidelberg, Germany

**2018:** Straub Young Scientist award for outstanding PhD student working in

Biological Research Centre, Szeged, Hungary

**Declaration by PhD supervisor and co-authors**

I declare that the data used in the thesis written by Ranti Dev Shukla reflect the contribution of the doctoral candidate to the article: "***Shukla, R.D**.; Zvara, Á.; Avramucz, Á.; Biketova, A.Yu.; Nyerges, A.; Puskás, L.G.; Fehér, T. inPOSE: A Flexible Toolbox for Chromosomal Cloning and Amplification of Bacterial Transgenes. Microorganisms 2022, 10, 236. https://doi.org/10.3390/ microorganisms10020236". The results reported in the PhD dissertation and the publication were not used to acquire any PhD degree previously. I further declare that the candidate has made a significant

contribution to the creation of the above mentioned publication.

Szeged, March 10, 2022

Tamás Fehér, MD, Ph.D

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Puskás, László, PhD