Scientific report for the period of October 2016 – September 2021

IS-silencing using CRISPR-interference

The long term goal of this work is the widening of our possibilities to use the simplest mobile genetic elements, bacterial insertion sequences (IS) for prokaryotic genome manipulation. The prerequisite of such use is the capability to precisely control IS mobility. Task 1 aimed at developing controllable downregulation of IS activity using CRISPR-interference (CRISPRi). CRISPRi allows the *in vivo* binding of a bulky protein, the 'dead'Cas9 (dCas) to virtually any DNA segment of choice, defined by a short piece of RNA, the CRISPR-RNA (crRNA). In this work, we designed and constructed a plasmid-borne CRISPR-array that expresses crRNAs targeting the left inverted repeats (IR) of IS*1*, IS*3*, IS*5* and IS*150* (Figure 1). The crRNAs were designed such that they overlapped with the predicted -10 box of the promoter of the respective transposase gene. We expected two consequences of dCas binding: downregulated transcription of the transposase and decreased transposition rate for each targeted IS. These phenomena were tested in parallel.

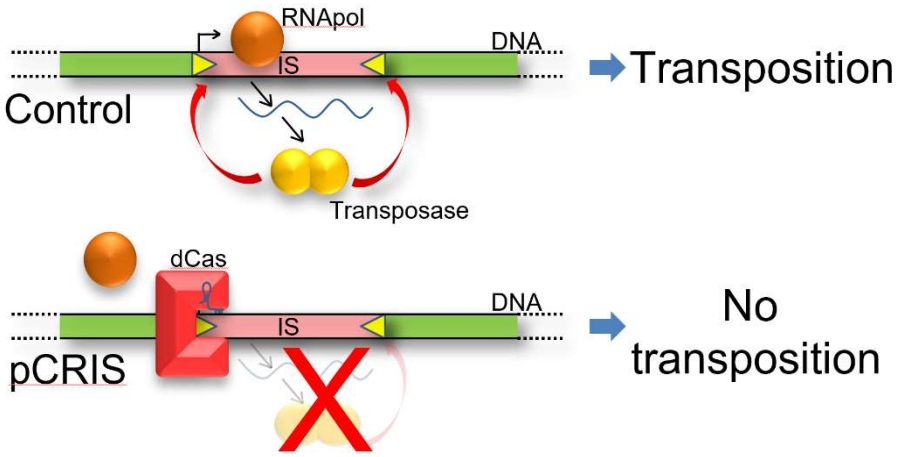

Figure 1. The theory of IS-silencing using CRISPR-interference

Transposase transcription levels were queried using transcriptome sequencing of control and IS-knockdown strains. The extent of downregulation was widespread: canonical IS*5* displayed more than 1000-fold silencing. IS*5*Y, IS*3* and IS*1*-variants were downregulated 1.6 to 51-fold, each case being statistically significant. The transcription of IS*150*, however did not change. The transposition rates of control and IS-knockdown strains were compared using two systems: one detects the spontaneous emergence of antibiotic-resistant cells, the other detects cells acquiring the capability of beta glycoside-assimilation. These systems were chosen since IS transposition is known to make a significant contribution to the rate measured by both. Both systems indicated a significant reduction in the rate of mutations, and classification of the detected mutants indicated the dramatic reduction of insertion mutants, i.e. the decline in the rate of IS transposition to the selected genomic loci.

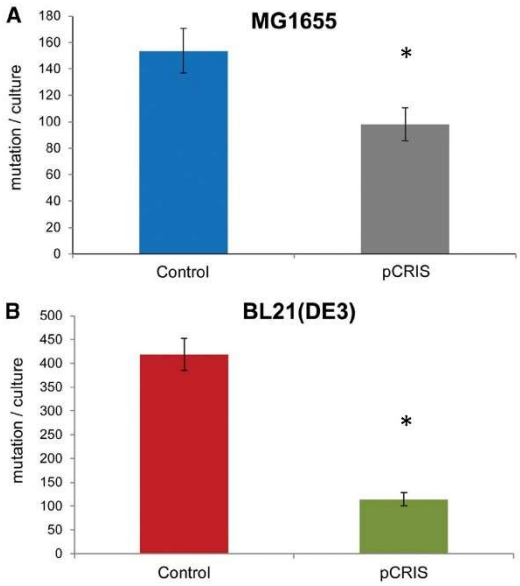

Figure 2. Effect of pCRIS-based IS-knockdown on the rate of *cycA* mutations in multiple *E. coli* strains: (**A**) *E. coli* K-12 MG1655 and (**B**) *E. coli* BL21(DE3). Error bars represent SD based on $n = 3$ independent measurements. *Significance ($P < 0.05$) based on *t*-test [1].

For the sake of future applications in the biotech industry, the effect of IS-silencing was also investigated on plasmid stability. After testing multiple plasmids, a construct expressing GFP from a bidirectional promoter was finally chosen, for it proved to be especially prone to IS-mediated inactivation. When monitoring the green fluorescence of induced cultures in evolutionary experiments, the half-life of the GFP signal was found to be extended if the GFP-construct was propagated in cells carrying the IS-silencing plasmid, as compared to the control. The dynamics of the loss of GFP fluorescence observed in numerous parallel cultures supported our hypothesis that the less frequent mutational inactivation of the *gfp* gene, and not the general facilitation of GFP function was responsible for the observed phenomenon. PCR analysis of the non-fluorescent cultures revealed that ISes inserted into the GFP construct were responsible for the loss of fluorescence.
Overall, we successfully decreased the transposition rate of four ISes to both genomic and plasmid-borne loci. The portability of the system was demonstrated by successful application in multiple *E. coli* strains. The transposition rate of all four ISes decreased, despite the fact that IS*150* was not transcriptionally silenced, indicating that the mere binding of the bulky CRISPR protein to the left IR is sufficient to downregulate transposition by sterically limiting the transposase enzyme's access to the locus [1].

Applying CRISPR-interference in *Synechocystis*
Having accumulated experience in the use of CRIPR-interference, I was invited to a project aiming to apply dCas9-based gene silencing in *Synechocystis* PCC 6803 for the first time. We targeted three genes involved in photosynthesis: *psbO,* encoding the manganese-stabilizing protein using two CRISPR-spacers, as well as *psbA2* and *psbA3* genes encoding the D1 subunit of photosystem II (PSII) using a single common spacer. The downregulation of all three genes was verified using qPCR and Western blot analysis. Based on the latter, the silencing of *psbO* was only partial, while the synthesis of both D1 subunit encoding proteins was completely eliminated. As a consequence, targeting *psbO* resulted in a 50% decrease of oxygen-evolution, while D1 silencing led to the complete loss of oxygen evolution as well as photoautotrophic growth arrest [2].

CRISPR/Cas-mediated engineering of bacteriophage genomes

In these two projects, we used our experience gathered in homologous recombination and CRISPR selection to carry out bacteriophage engineering, *in vivo*. The first task was to label the K1F phage with a *gfp* gene. K1F phage can lyse *E. coli* K1, a dangerous pathogen causing neonatal meningitis or adult urinary infections. K1F has the potential to provide phage therapy for patients infected by *E. coli* K1. However, the use of bacteriophages is not permitted for routine human therapy in most western countries, due to the lack of fundamental biological data on the mechanisms of phage action inside a human host. Our aim was to generate GFP-labeled K1F to aid cell biology studies applying *ex vivo* phage therapy models, taking place at the University of Warwick. Phage engineering required generating donor DNA plasmids, harboring the *gfp* gene flanked by appropriate homology arms. Growing phages on *E. coli* harboring the donor plasmid allows the *gfp* to insert into the phage genome by recombination, and results in a mixed phage lysate, consisting of WT and recombinant phages. By growing the phage mixture on a selection strain of *E. coli*, which harbors pCas9 plasmids targeting the WT genotype, one can enrich the recombinants within the phage mix. We applied this strategy to direct the GFP to the N- or the C-terminus of the major capsid protein, or to the C terminal of the minor capsid protein. Although recombinants were detectable in all three cases, enrichment by CRISPR selection was only successful in the case of the C-terminally labelled minor capsid protein. In the end, we were able to obtain pure clones of K1F*g10b::gfp*, carrying C-terminal translational fusions of the minor capsid protein and GFP. This phage, when applied in sufficient numbers, can be visualized under a light microscope using GFP-enhancer antibodies. The microscopic investigations, carried out by our collaborator in the UK uncovered multiple important phenomena taking place during the model phage therapy process (Figure 3). For example, urine bladder epithelial cells were shown to be capable of phagocytosing both bacteria and phages in independent processes. Phages were found to dock to bacteria inside intracellular vacuoles, followed by the killing of bacterial cells. Bacteria escaping the phagosomes were degraded by xenophagy, which was further potentiated by the presence of bacteriophages. These observations could, in the long run contribute to the better understanding of phage therapy and support its more widespread use to fight bacterial infections in the future [3].

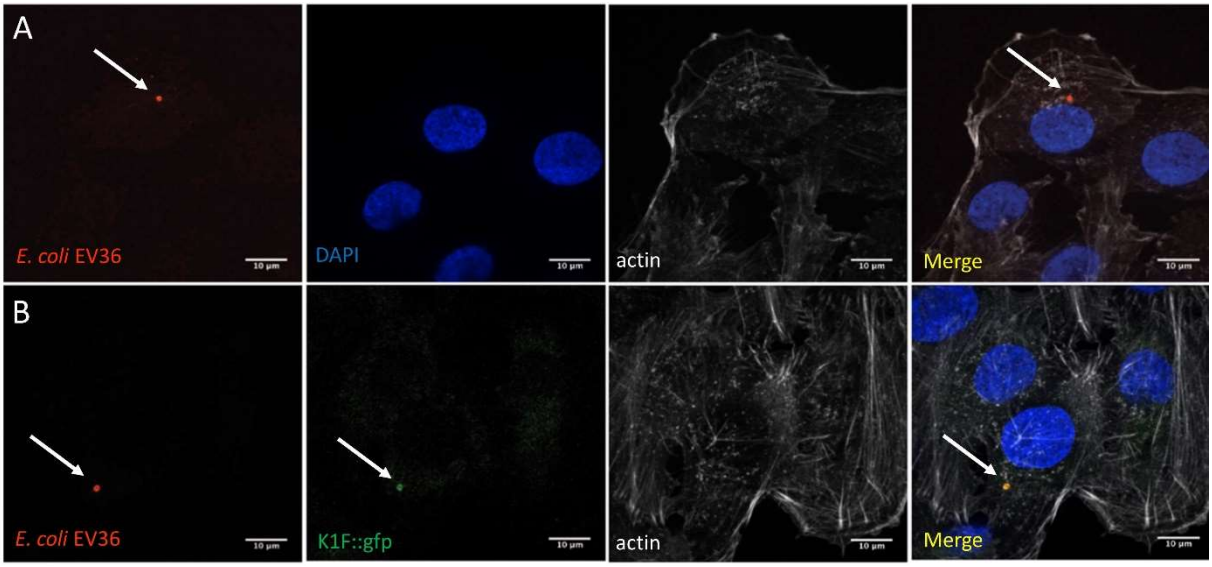

Figure 3. Phage K1F targets extracellular and intracellular bacteria in epithelial human cells. Confocal imaging and quantification of intracellular *E. coli* EV36-RFP and fluorescent phage K1F in human epithelial cells. T24 human urinary bladder epithelial cells have been stained with Phalloidin as a marker for F- actin and DAPI for DNA-rich nucleus. Each panel represents one channel of a single image. 'Merge' panel shows all channels merged into one image. Each set of panels come from a single image. (**A**) T24 cells have been infected with *E. coli* EV36-RFP and further incubated with gentamycin to ensure the clearance of extracellular bacteria. The arrow indicates intracellular *E. coli* EV36-RFP bacteria. (**B**) T24 cells have

been infected with*E. coli* EV36-RFP and fluorescent phage K1F*g10b::gfp* has been added. The arrows indicate intracellular *E. coli* EV36-RFP and intracellular fluorescent phage K1F co-localising [3].

In our second phage engineering work, our task was to engineer a hybrid T7 bacteriophage that harbors the receptor binding proteins of the K1F phage. Such re-tuning of phage T7 host specificity (from K12 to K1) could be an important process in future potential phage therapy protocols when a well-established, thoroughly studied and precisely controlled phage, such as T7 should be quickly redirected towards a pathogenic bacterium that is not normally within its target range. Two phage editing strategies were applied to exchange the genes of the receptor-binding proteins of T7: plasmid-based homologous recombination, and bacteriophage recombineering using electroporated DNA (BRED). After either recombination process, multiple ways of phage selection were tested to obtain a pure recombinant phage, including *trxA*, host-specificity, and CRISPR-Cas-based selection. Although a stable phage displaying host switching was not assembled in this work, the large clear plaques formed on K1-expressing *E. coli* cells by the phages obtained using BRED were a strong indication that the necessary genes required for serial lysis of a non-canonical host were all present. The use of BRED for this purpose could therefore be a quick way in the future to test the potential use of a new tail fibre without the need to engineer a stable phage [4].

Using bacterial IS elements for transgene integration and amplification

Today, the fastest and simplest way to maintain and/or express a gene or operon of interest is to clone it into a bacterial plasmid, usually choosing one capable of replication in *Escherichia coli*. Protein expression from plasmids however, may significantly alter the physiology of the host cell, especially in the case of high copy-numbers, thereby derailing fundamental studies. Plasmids also require constant selection, often warranted by antibiotics and their respective resistance genes. From an industrial viewpoint, this not only poses an additional cost due to the administration of the antibiotic, but also due to the need of its removal from the final fermentation product. In addition, secreted resistance factors, like the β-lactamase enzyme allow plasmids to be lost from cells during selection, leading to inhomogeneous cell population. A similar impairment of experimental reproducibility can be caused by variations in plasmid copy numbers. These factors all indicate the necessity to develop techniques permitting transgene insertion into the bacterial chromosome.

To address this issue, our task was to test the use of IS elements as targets for recombination-mediated insertion of linear DNA fragments into the bacterial chromosome. The goal was to develop a system that relies on the multiple copies of IS elements within the chromosome to rapidly insert 'cargo' genes (or transgenes) into the genome in multiple copies (Figure 4). In our experiments, we attempted the use of IS*1*, IS*2*, IS*3* and IS*150* elements as targets, or 'landing pads'. The recombination process was mediated by temporarily expressing the λ-Red recombinases within the target *E. coli* cell. We successfully integrated antibiotic-resistance genes into all four types of IS elements listed above, verifying that they can be used as landing pads for single-copy transgene entry. By testing multiple resistance genes, we found that Spectinomycin and Kanamycin-resistance (SpR, KmR, respectively) genes are the best choice to use as selection markers due to providing high efficiency of recombination and low rate of false positive colonies. In an attempt to increase the rate of transgene insertion, we applied simultaneous CRISPR/Cas cleavage of the landing pad in vivo, to increase recombination efficiency by generating free DNA ends. This technique (tested for IS*1* and IS*3*) meant the co-transformation of cells with the linear DNA and the pCas9 plasmid targeting the respective IS element. Unfortunately, it did not lead to a straightforward increase in transgene insertion rate in either case, most possibly due to the generally low frequency of co-transformation.

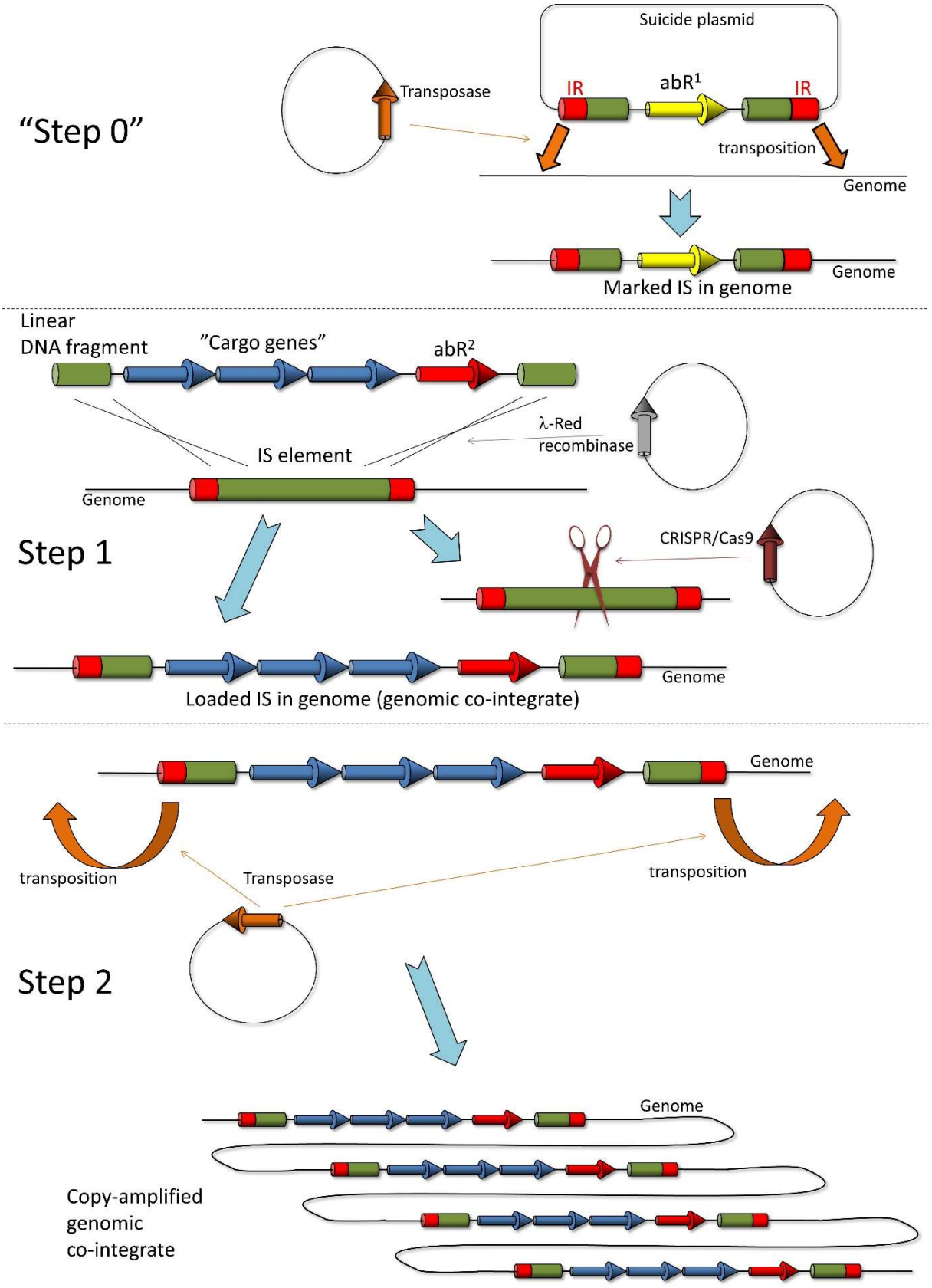

Figure 4. The inPOSE protocol. Step 1 is the entry of a gene or operon of interest into an IS element residing on the host genome by recombineering, facilitated by the λ-Red recombinase enzymes. CRISPR/Cas-mediated cleavage of the wt IS element(s) enforces selection for the recombinants and (if applied concomitantly) facilitation of the recombination event. In Step 2, the genomic co-integrant (i.e. the loaded IS) is copy-amplified by the transposase corresponding to the IS, expressed

in trans. “Step 0” is an optional accessory step that can transpose the marked IS element into the genome of host cells chosen for chromosomal transgene cloning. Green boxes: the targeted IS element (or segments thereof); red boxes: inverted repeats (IRs) of the IS; yellow and red arrows: two different antibiotic resistance genes (abR^1 and abR^2, respectively); blue arrows: the transgenes to be integrated; orange arrows: transposase gene of the targeted IS; gray arrow: λ-Red recombinase genes; brown arrow: CRISPR/Cas genes; open thin black lines: host bacterial genome; closed thin black lines: circular plasmids.

A more advanced application of the IS-mediated entry of transgenes into the bacterial chromosome is the potential integration of the cargo gene into multiple copies of the same IS element in a single step. This was attempted using IS*1* or IS*3* as targets, applying *E. coli* strains that harbor two copies of the respective IS elements. In such cases the antibiotic resistance marker is insufficient to select for double integration events. We therefore facilitated the antibiotic selection with CRISPR/Cas selection. This meant the transformation of the pCas9 plasmid targeting the respective IS element after Spectinomycin selection for the entry of linear DNA fragments. The CRISPR/Cas cleaved the wild type IS *in vivo*, which is lethal to the cell but left cells intact if both copies of the respective IS were loaded with the transgene. We successfully integrated two copies of the SpR gene in a single step using this protocol, and demonstrated the feasibility of this strategy both with IS*1* and IS*3* serving as landing pads.

Another application of CRISPR/Cas-mediated genome editing is the integration of non-selectable genes into the bacterial chromosome. We successfully integrated the gene of Green Fluorescence Protein gene (*gfp*) into the chromosome of *E. coli* using a single copy of IS*3* as a target. The CRISPR/Cas9 system was expressed subsequently to the recombineering using the pCas9IS3 plasmid. Attempts to target IS*1* were unsuccessful with this system. However, applying Cas9 cleavage concomitantly to the recombineering step (using pKDsg-IS1 and pCas9cr4 plasmids) permitted the integration of *gfp* into IS*1*. Both genomic co-integrant strains of *E. coli* displayed significantly increased green fluorescence compared to the maternal strains, as detected in a microplate reader.

The main reason for choosing IS elements as landing pads for transgene integration is to allow their subsequent copy-number amplification by replicative transposition (Figure 4). We wished to accomplish this amplification by overexpressing the transposase gene of the respective IS element *in trans*, we therefore generated inducible expression plasmids carrying the transposase genes of IS*1* and IS*3*, respectively. The cloning process was designed in a way to attain the fusion genes (orfAB) on the plasmids, lacking the need for a translational frameshift for the synthesis of the functional enzyme protein. For IS*3*, the codon-optimized version of orfAB was also commercially synthetized. The effect of overexpressing these constructs was tested on IS*1*::SpR and IS*3*::SpR containing cells. After induction of the transposase, the cells were plated on Spectinomycin-gradient plates to select for cells displaying increased resistance due to copy number-amplification of the resistance cassette. For IS*3*, only the synthetic version of the transposase gene was found to be active. The obtained highly resistant cells were verified to contain multiple copies of the resistance gene using droplet-digital PCR (ddPCR). For IS*1*, the transposase was found to be active using outcrossing and gradient-plates, but the reproducibility of these experiments was low due to an unknown factor. For this reason, our further amplification experiments mostly utilized IS*3* elements carrying the inserted genes of interest.

To simulate the industrial application of our strategy (which we refer to as inPOSE), we integrated a five-gene operon (vioABCDE) into the genomic IS elements as well. VioABCDE encodes the enzymes responsible for the synthesis of violacein, a purple pigment originally produced by *Chromobacterium violaceum*. The clear advantage of using vioABCDE is the possibility of constantly monitoring the functionality of the operon. Integration of vioABCDE marked with a resistance gene into either IS*1* or IS*3* was readily obtained using λ-Red-

recombineering, however, the purple color was lost when targeting IS*1*. When applying concomitant CRISPR/Cas cleavage with the recombineering process (using pKDsg-IS1 or pKDsg-IS3 and pCas9cr4 plasmids), the 9 kbp-long cassette could be inserted into IS*1* or IS*3* targets in two copies, too. Most importantly, the vioABCDE cassette (marked with a resistance gene) could also be copy-amplified by the induction of the corresponding transposase enzyme. The violacein production of the colonies displaying increased resistance could be monitored simply by visual inspection (Figure 5).

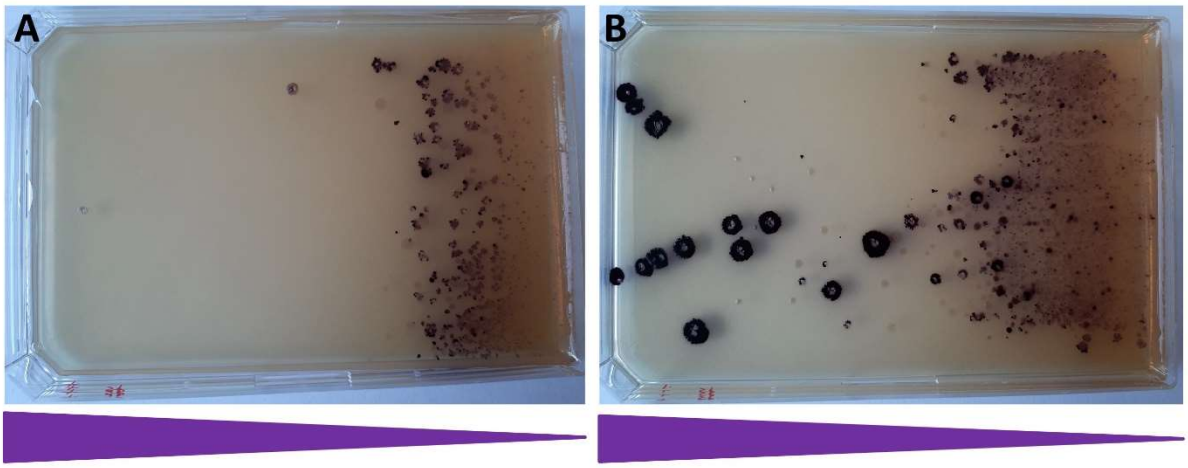

Figure 5. The effect of one round of IS*3* transposase induction on the Sp-resistance of BLK09IS*3*::VioABCDE_SpR (locus 2) colonies. Note the intense purple colors of the colonies caused by violacein. (A) Uninduced cells, (B) cells induced with aTc. The triangles represent the gradient of Sp concentration within the medium.

The copy-amplification of the IS3::vioABCDE cassette was tested in multiple *E. coli* strains: BL21(DE3) derivate multiple-deletion strains (BLK09 and BLK16), and K-12 derived multiple deletion strains (MDS27 and MDS30). Even a single round of transposase induction led to the appearance of colonies harboring multiple copies of the inserted cassette, as verified by ddPCR. Amplification also worked when using KmR as a resistance marker. To test the upper limits of copy-number amplification of the modified IS*3*, we repeated the induction process for three rounds in BLK16_IS*3*::vioABCDE_SpR, analyzing the obtained colonies after every round. The results, shown in Figure 6 indicate that the first two rounds of transposase induction lead to a significant increase in the mean copy number of the SpR gene, as analyzed by ddPCR. In the third round, there is no further increase of the mean, probably due to the potential fitness cost of carrying a higher number of modified IS3 elements on the chromosome. One can nevertheless find colonies carrying increased IS3 copy numbers as a result of the third round of induction, but they are counterweighed by lines that have lost IS*3* copies during the third induction process.

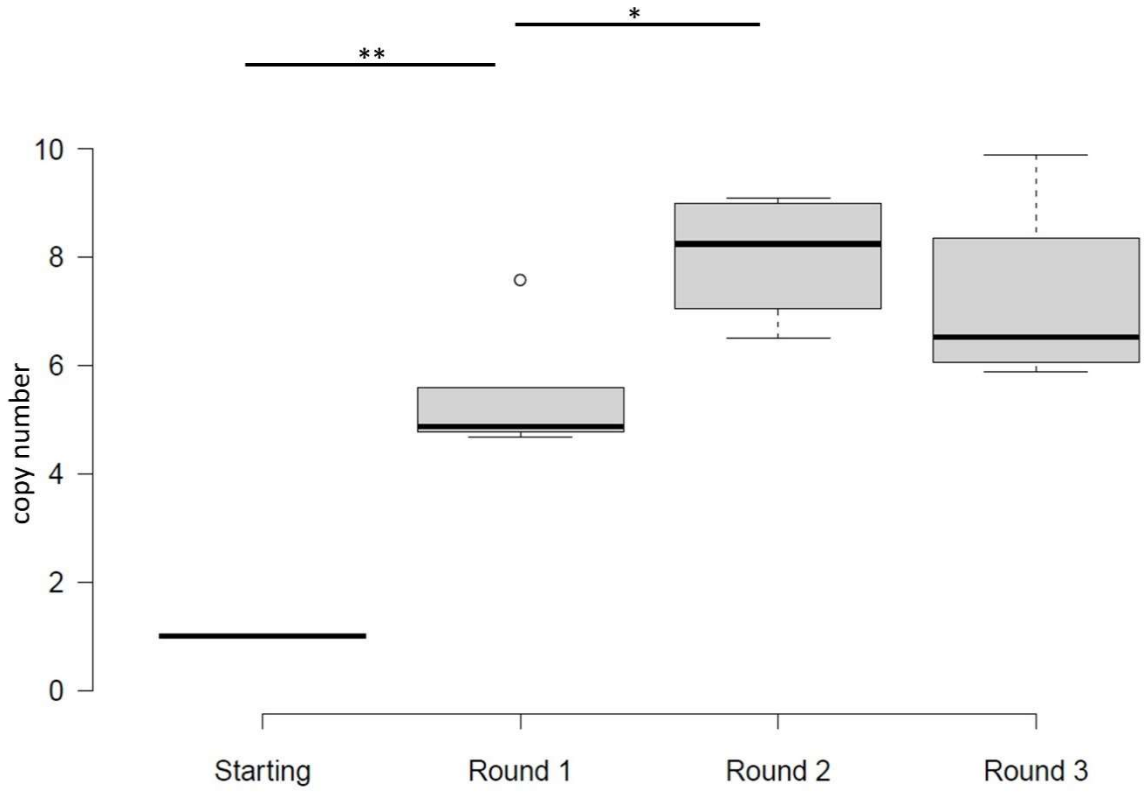

Figure 6. Copy numbers of the SpR gene of *E. coli* BLK16_IS*3*::VioABCDE_SpR detected by ddPCR after 1, 2 or 3 rounds of IS*3* transposase induction. Center lines show the medians; box limits indicate the 25th and 75th percentiles as determined by R software; whiskers extend 1.5 times the interquartile range from the 25th and 75th percentiles, outliers are represented by dots. n = 1, 5, 4, 4 sample points. * p<.05 with two tailed, unpaired t-test; ** p<.002 with two tailed, one sample t-test.

We analyzed several purple colonies from the three rounds of induction by ddPCR, and chose the ones displaying the highest copy numbers for quantitative analysis of violacein content. As apparent on Table 1. the violacein content showed a roughly acceptable correlation with the SpR copy number determined by ddPCR. After round 3 however, the 26 copies of SpR, implied by ddPCR did not match the relative violacein content of 13.52 units (compared to the 1 unit of the starting strain).

Table 1. Properties of *E. coli* BLK16IS*3*::VioABCDE_SpR derivatives after the indicated rounds of IS3 transposase induction

Strain code	**Strain name**	**SpR copy number by ddPCR**	**Violacein content (AU)**	**Relative violacein content[1]**	**Relative sequence coverage of VioABCDE by WGS[2]**
B0	BLK16IS3::VioABCDE_SpR, starting	0.98	0.06	1	1
B1	BLK16IS3::VioABCDE_SpR, 1st round	5.59	0.2926	4.88	5.71
B2	BLK16IS3::VioABCDE_SpR, 2nd round	9.10	0.7847	13.08	11.32
B3	BLK16IS3::VioABCDE_SpR, 3rd round	26.11	0.8113	13.52	12.51

SpR: Spectinomycin resistance
ddPCR: droplet digital polymerase chain reaction
AU: arbitrary unit, based on OD_{585} measurement

WGS: whole genome sequencing
[1] Relative to strain B0
[2] Ratio of sequence reads obtained +/- 10 nt of peak border

To relieve this contradiction, we carried out whole genome sequencing (WGS) of the starting strain (B0) and the strains obtained upon the three rounds of induction (B1, B2 and B3). The obtained copy number in strain B3 (13.52) supports the violacein level, not the ddPCR value. In fact, the correlation between the violacein levels and the IS*3*::vioABCDE_SpR copy numbers determined by WGS was very strong (R^2=0.98). Besides verifying the ddPCR results, we used our WGS data to i) to analyze the fraction of mutant or truncated VioABCDE operons, ii) to test whether amplification leads to tandem repeats or a random scatter of the loaded IS*3* element within the genome and iii) to check for unexpected genomic rearrangements. Positively, we found no vioABCDE operons that were truncated or mutated. However, the IS3::vioABCDE_SpR elements were not randomly scattered in the genome, but were forming a special type of tandem repeat: the modified IS*3* elements were amplified along with the 14-18 kbp-long genomic sequence lying directly downstream to their right end, therefore the IS*3* cassettes were not lying back-to-back, but were separated by the co-amplified 14-18 kbp segment.
Finally, to determine whether chromosomal expression of the vioABCDE operon had any advantage compared to the plasmid-based expression of the same operon, we carried out two experiments to monotor violacein production in the lack of antibiotic selection. The first type of experiment monitored the ratio of purple colonies in the lack of antibiotic selection. As apparent on Figure 7, the purple color was rapidly lost from colonies originally carrying plasmid-based vioABCDE. On the contrary >96% of the colonies of the genomic cointegrants maintained purple color in the lack of selection, even after 50 generations of culturing.

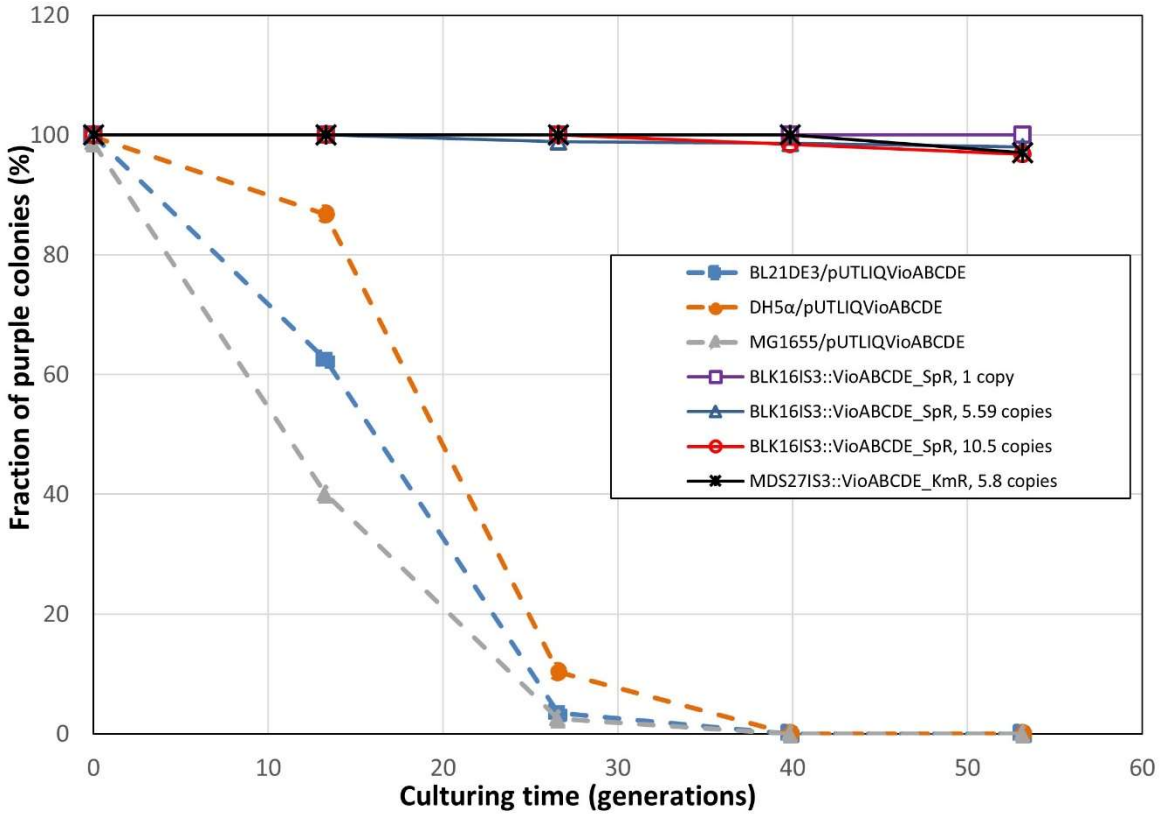

Figure 7. Fractions of violacein-producing cells within bacterial cultures grown in the lack of antibiotic selection. Dashed lines mark strains carrying the pUTLIQvio_ABCDE plasmid, solid lines mark strains carrying an IS*3*::vioABCDE cassette on their chromosomes, as indicated on the legend. All values are means of three biological replicates.

The second type of experiment monitored violacein production of liquid cultures grown in the lack of selection. Similarly, violacein levels rapidly decreased for strains expressing the vioABCDE operon from plasmids, while they displayed no significant decrease upon 40 generations of culturing when expressed from the chromosome (Figure 8).

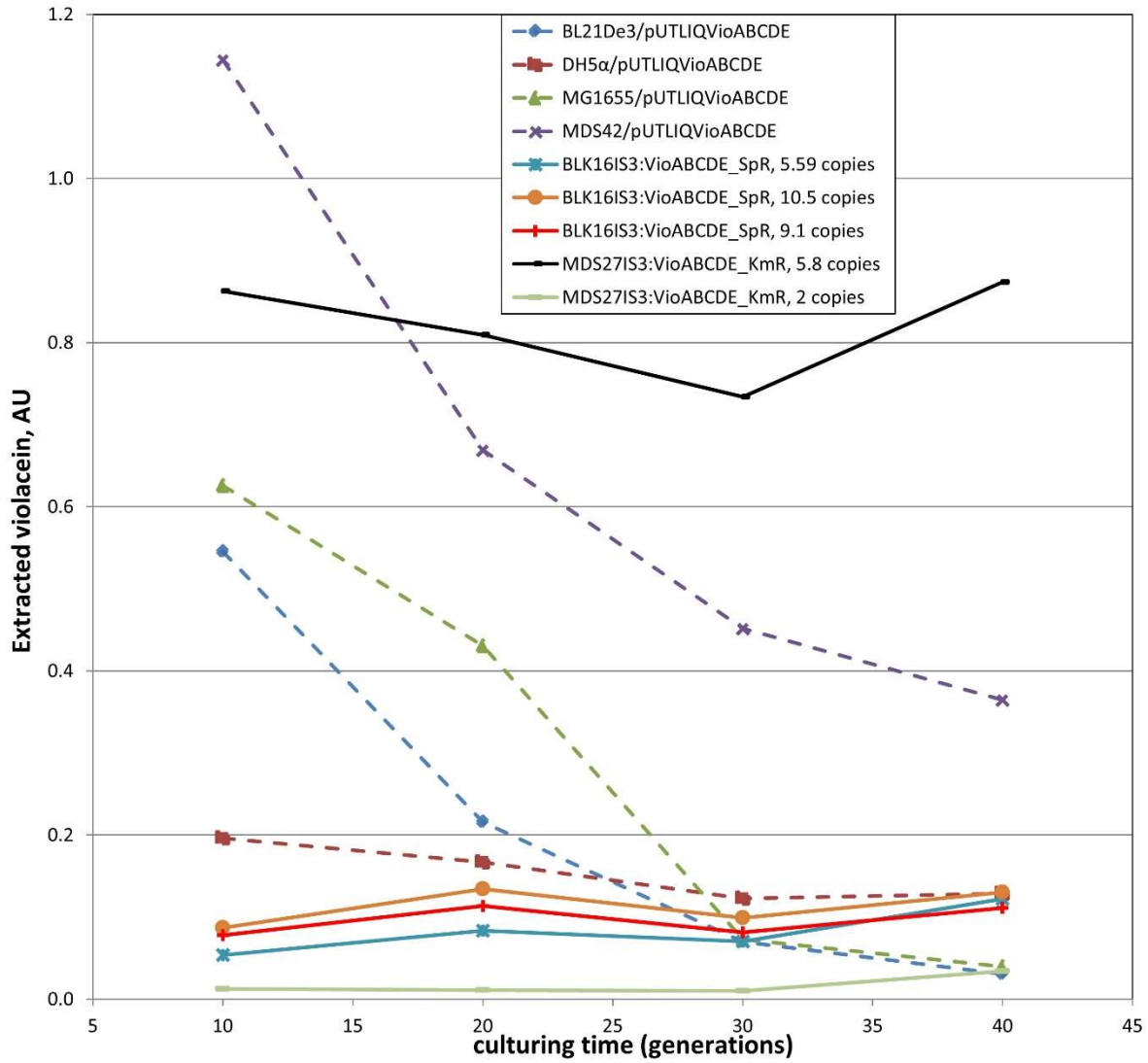

Figure 8. Violacein levels of liquid bacterial cultures grown in the lack of antibiotic selection. Dashed lines mark strains carrying the pUTLIQ_vioABCDE plasmid, solid lines mark strains carrying an IS3::vioABCDE cassette on their chromosomes, as indicated on the legend. All values are means of three biological replicates.

The utility of the inPOSE system for transgene entry by homologous recombination, and transgene amplification by copy/paste transposition was demonstrated in the experiments above. We have no reason to think that IS*3* is the only element applicable for this purpose, nor is it necessarily the best. In fact, we only refer to inPOSE as a strategy, not a discrete tool, and believe that other mobile elements should be tested for this purpose to be applied in further

bacterial strains or species. For those future users, who wish to use IS*3* for this purpose, but their targeted bacterial cell lacks this element, we showcase a transposition-based method to insert a marked copy of IS*3* into the genome of the chosen host bacterium. We refer to this as "Step 0" of the inPOSE protocol, which can be readily continued with Step 1 if using a different antibiotic for transgene insertion (Figure 4).
Overall, in this project, we have shown successful genome editing of *E. coli* by recombineering targeting IS*1* or IS*3* elements, using CmR, SpR or KmR as selection markers. We have demonstrated integrating solely a resistance gene or a five-gene operon marked with a resistance gene this way. Facilitating the recombineering step with CRISPR/Cas cleavage (applied either concomitantly or subsequently) aided the integration of long (>9 kbp) cassettes and was essential to insert unselectable genes (e.g. *gfp*) or to obtain double co-integrants of selectable gene cassettes in a single step. We have displayed the reproducible and controllable copy-number amplification of IS*3*-carried transgenes by expressing the respective transposase in trans. Our work also demonstrated the increased stability of chromosomal transgenes in the lack of selection, compared to their plasmid-borne counterparts. The flexibility of this strategy, called inPOSE, is showcased using the IS*3* element, but is most likely applicable to other bacterial ISes as well. For those who wish to use IS*3* for this purpose in strains harboring no IS*3* copies, we developed tools to rapidly introduce a marked copy of IS*3* into the chromosome by transposition.
Should the reviewer be interested in all details concerning this final work, we recommend accessing our manuscript, deposited to BioRxiv [5].

Addressing the objectives of the grant proposal

The specific questions addressed in my grant proposal were the following:

1. Can the copy number of certain TEs be dramatically increased using hyperactive transposase expression? Yes.
2. Can forces of selection alone limit the uncontrolled replicative transposition of a hyperactive TE? If yes, what is the limit of TE copy number in *E. coli*? Yes, and in our experiments dealing with long (>9kbp) inserts into IS*3* elements, the mean copy numbers did not increase above 8. By analyzing individual clones after the third round of induction, we were nevertheless able to find clones harboring 13 copies of the modified IS*3*. We suspect that finding clones with more than 20 correct copies would require screening an extensive number of colonies, if possible.
3. Can transposition frequencies be decreased via transcriptional silencing of the transposase genes? Yes.
4. How are genomic stability, cellular fitness and evolvability affected by an increase in TE copy number? Our data is limited concerning this question. In the lack of selection, cells harboring multiple copies of the IS*3*::vioABCDE_SpR element nevertheless displayed increased stability of violacein production, compared to plasmid based production. Due to the tandem repeats formed by the amplified IS elements however, we suspect that in the long run, some of these modified elements could be lost by homologous recombination.
5. How are genomic stability, cellular fitness and evolvability affected by transposase-silencing? We measured increased genetic stability at chromosomal and plasmid-based loci, alike.
6. How does the genetic content of TEs affect their mobility and upper limit of copy number? Our dataset is incomplete, we however believe that carrying a long, functional operon encoding an enzymatic cascade has a severe negative effect on fitness with increasing copy numbers.
7. Can TEs serve as targets for the insertion of useful genetic constructs into the genome? Yes.
8. Can TEs be used for the scalable amplification of useful genetic constructs? Yes.

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