Investigating Precatenation During DNA Replication in *E. coli*.

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Introduction

Speculating about the topological problems of DNA replication, Watson and Crick observed that "although it is difficult to see at the moment how these processes occur without everything getting tangled, we do not think that this objection will be insuperable" (WATSON and CRICK, 1953). It is now known that topoisomerases function to resolve topological problems and maintain desired DNA topology in cells, by making breaks in DNA, through which strands are passed, before religation (Champoux, 2001). In prokaryotes, it is DNA gyrase that acts ahead of the replication fork to relieve positive supercoiling accumulating as a result of strand unwinding, allowing progression of DNA replication.

Within the last 200 bp of a prokaryotic chromosome, as replication forks converge however, there may be no space for topoisomerases to access DNA (Schvartzman et al., 2019). Champoux and Been (1980) have suggested swivelling of the replisome would diffuse positive supercoiling into the replicated region as precatenanes, linking newly replicated sister chromatids as catenanes upon completion of replication. If precatenanes are indeed formed, type II topoisomerases such as TopoIV would be able to act on the replicated region to remove final linkage between the two product chromosomes (Hiasa and Marians, 1996). Additionally, findings that TopoIV knockout strains were viable with overexpression of Topoisomerase III, also implicated the type I topoisomerase, TopoIII, in a role in chromosome segregation (Perez - Cheeks et al., 2012).

The question remains whether precatenanes are able to form during replication. Findings have both supported and contradicted their formation, with doubt over whether the replisome would be able to swivel to create precatenation (Peter et al., 1998). There have been claims of precatenanes detected in replicating plasmids, both by electrophoresis and electron microscopy, supporting the proposition of Champoux and Been (Peter et al., 1998; Cebrián et al., 2015), however, electron microscopy, has also shown replication intermediates with no linkage in the replicated region (Fuke and Inselburg, 1972).

To analyse catenanes within the chromosome of *E.coli,* recombination by ϕC31 integrase at *attP-attB* sites has been previously used by the Colloms lab, where excision on catenated sister chromatids would lead to the production of two linked cassettes, while recombination in the absence of

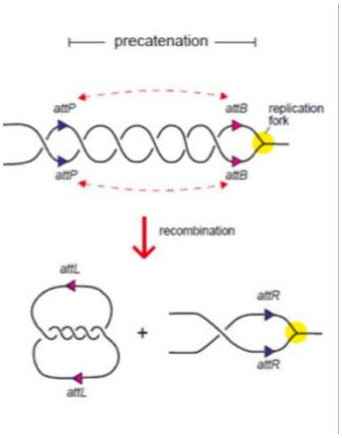


Fig 1: Catenane capture by ϕC31 integrase-catalysed cassette excision. (From Tolmatcheva, 2019)

catenation would produce two unlinked cassettes (Fig 1). However, to date, no precatenanes have been reliably detected by this method.

Aims

The first aim was to take on the problem of whether catenanes are formed during DNA replication in prokaryotes or not, using recombination between *attP* and *attB* sites to capture catenanes in *E.coli's* chromosome, working in a *topB* (TopIII knockout) background, where precatenanes would be expected to persist longer.

The second aim was to construct chemically inducible ϕC31 integrase, using a split position at amino acid 571, as trialled by Weinberg *et al.* (2019), and rapamycin-dependent dimerization domains FKBP and FRB. This was intended to eventually improve integrase regulation in the catenane capture assay.

Materials Methods

Table 1: Strains list. Both *E. coli* strains used, and strains from which these were derived, are listed. Those shaded in blue have been made in this investigation by P1 transduction.

Strain	**Properties**
C600	*thr*-1 *leuB6*(Am) *fhuA21 cyn*-101 *lacY1 glnX44*(AS) λ^- *e14*$^-$ *rfbC1 glpR200*(glpc) thiE1
C600S	C600 *recA* Str^R
C600SN	C600S *gyrA*^{S83L} Tn10 TetR
C600SN ::tam	*attP – attB* cassette replacing *tam*
W1485	F$^+$ *glnV42*(AS) *λ- rpoS396*(Am) *rph*-1
CB0129	F$^-$ W1485 *thyA leu deoB or C supE*
LN266	CB0129 *rpsL2666* (Str^R)
CP100	LN266 *ΔlacI*
CP100 dif+	*attP – attB* cassette between *dif* and *yne1*
CP100 Δdif	*attP – attB* cassette replacing *dif*
CS1	CP100 *dif+ attP - attB topB* ::Gent^R
CS2	CP100 *Δdif attP - attB topB topB* ::Gent^R
DH5∝	*Ø80d lacZΔM15 endA1 recA1 hsdR17* (r_k^-,m_k^+) *phoa supE44 λ- thi-1 gyrA96 relA1* F$^-$ *Δ(lac ZYA- arg F) U169*
AB1157	F- *thr-1 araC14 leuB6*(Am) *Δ(gpt-proA)62 lacY1 tsx-33 qsr'-0 glnX44*(AS) *galK2*(Oc) *λ- Rac-0 hisG4*(Oc) *rfbC1 mgl-51 rpoS396*(Am) *rpsL31*(Str^R) *kdgK51 xylA5 mtl-1 argE3*(Oc) *thiE1*
DS941	AB1157 *recF lacI*^{q} *lacZΔM15*
DS941 dif+	*attP – attB* cassette between dif and yne1
DS941 Δdif	*attP – attB* cassette replacing dif
DS941::tam	*attP – attB* cassette replacing tam
CS3	DS941 *dif+ attP - attB topB* ::Gent^R
CS4	DS941 *Δdif attP - attB topB topB* ::Gent^R
CS5	DS941 *::tam attP - attB topB* ::Gent^R
CS6	DS941 *dif+ attP - attB topB* ::Gent^R *Gyra*^{S83L} (Tn10, TetR)
JAM107 TopB ::GentR	DS941 *topB* Gent^R

Strain Construction by P1 Transduction:
Liquid cultures of strains to be transduced were grown overnight in L-broth, then diluted 1/50 and grown for a further 90 min, approximately to mid-log phase. 1.5 ml culture was centrifuged (5000 xg, 2 min), and resuspended in 500 µl L-broth, 500 µl 50 mM $CaCl_2$, and 500 µl 100 mM $MgCl_2$. P1 lysate, prepared on JAM107 *topB* ::$Gent^R$, was added. Samples were incubated at 37 °C for 30 min, and infection was stopped by addition of 150 µl 1 M sodium citrate. This was followed by two cycles of centrifugation at 5000 xg, 2 min, and resuspension in 1 ml L-broth with sodium citrate up to 100 mM. Samples were then incubated at 37 °C for 1 hour, shaking. A last round of centrifugation and resuspension in 200 µl L-broth with sodium citrate up to 100mM was carried out, and finally, 200 µl of each sample was plated on L-agar supplemented sodium citrate up to 10 mM, and 10 µg/ml gentamycin to select for *topB* mutants. Transductant colonies were re-streaked three times on L-agar with sodium citrate and gentamycin, and deletion was confirmed by PCR.

Catenane Capture:
Overnight cultures of strains containing the *attPB* kan^r *lacI* cassette and plasmid pZJ7m, encoding ϕC31 integrase under an arabinose-inducible promotor, were grown to an OD_{600} of approximately 1. Cells were harvested by centrifugation at 6000 x g for 3 minutes, resuspended in 50 ml of L-broth and split into 4 aliquots. One aliquot had no further additions, one received 0.2 % arabinose to induce integrase expression, 10 µg/ml norfloxacin was added to the third, and 0.2 % arabinose was added to the fourth aliquot, followed by norfloxacin 2 min later. After growth for 30 min, at 37 °C, shaking, covalently closed circular DNA was extracted from samples using Qiagen QIAprep Spin Miniprep kit.

Southern Blot:
DNA samples from above were run on a 1 % agarose gel in TAE buffer. Standard protocols were used for Southern blot (Amersham Hybond-N+ Product Manual), with probes complementary to the kanamycin resistance gene on the attPB cassette (from plasmid pUC71K) used for hybridisation, labelled according to Thermo Scientific Biotin DecaLabel DNA Labelling Kit. Chemiluminescent detection was according to instructions for Thermo Scientific Chemiluminescent Nucleic Acid Detection Module. Chemiluminescence was detected using Fusion FX camera.

Plasmid Construction:
Plasmids encoding fragments of the chemically inducible split ϕC31 integrase were constructed as shown in Fig 2, using standard molecular cloning techniques.

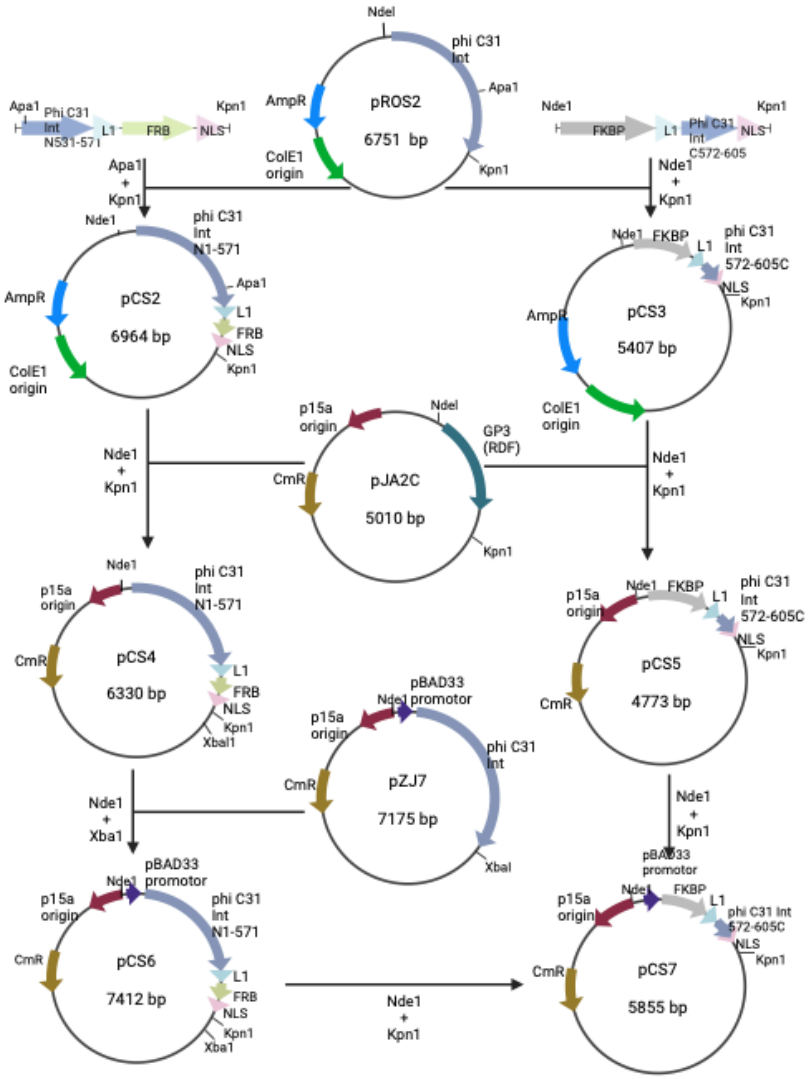


Fig 2: Construction of a set of plasmids encoding rapamycin inducible split ϕC31 integrase. G-blocks (synthetic DNA fragments) encoding integrase fragments, joined by linker L1 to the dimerization domains FRB or FKBP, as well as the nuclear localisation signal (NLS) SV40, were initially inserted into vector plasmid pROS2. pCS2 had an N-terminal integrase fragment from amino acid 1 - 571, L1, FRB and the NLS. pCS3 encoded FKBP, L1, integrase from amino acid 572 to 605, and the NLS. pJA2C served as a as further vector for construction of pCS4 and 5, with the same inducible integrase fragments, but different origins of replication and antibiotic resistance, to allow transformation with plasmids encoding both integrase fragments. Finally, pCS6 and 7, derived from pZJ7 with the pBAD33 promoter, had arabinose inducible expression of integrase fragments. (Image created in Biorender.com)

Testing activity of Split-Integrase: transformation

pCS3 (FKBP – L1 – ϕC31 Int C572-605 – NLS) and pCS4 (ϕC31 Int N1 – 571 – L1 – FRB – NLS) were transformed into CP100 *dif*$^{+}$ *topB*. Transformants were plated on L-agar containing X- gal (2 µl per 1 ml agar), 0.3 M thymidine (required by CP100) and selective antibiotics, both with 10 µM rapamycin and without.

Testing activity of Split-Integrase: time-course

A time-course of recombination after addition of rapamycin was carried out. Overnight cultures of transformants above were diluted 1/40 into 20 ml L-broth (with 25 µg/ml kanamycin, 0.3 M thymidine, 10 µg/ml ampicillin and 25 µg/ml chloramphenicol) and grown until approximately mid-log phase. Samples were centrifuged (6000 xg, 3 min) and resuspended in 20 ml L-broth, without kanamycin. 100 µl was sampled before treatment, and at time intervals 0, 15, 30 and 60 min after

addition of rapamycin to a final concentration of 10 µM. Samples were immediately centrifuged (13000 rpm, 2 min) to remove rapamycin, before resuspending and diluting 10^{-5} in L-broth, plating 200 µl on L-agar with X-gal, thymidine, chloramphenicol and ampicillin.

Testing activity of Split-Integrase: rapamycin concentration

Activity of pCS3 and pCS4 with varying rapamycin concentrations was similarly investigated, growing cultures for 30 min in different dilutions of rapamycin before plating on X-gal.

Testing activity of Split-Integrase: arabinose inducible expression:

Overnight cultures of CP100 *dif*$^{+}$ *topB*, transformed with pCS3 (FKBP – L1 – ϕC31 Int C572-605 – NLS) and pCS6 (ϕC31 Int N1 – 571 – L1 – FRB – NLS, arabinose inducible expression) were grown until an OD_{600} of approximately 1, in glucose to repress expression of integrase on pCS6. Culture was centrifuged (600 xg, 3min) and resuspended in 40 ml L-broth without glucose. This was split into four 10 ml aliquots, with one receiving no treatment, one 0.2 % glucose, one 0.2 % arabinose to induce expression of integrase, another glucose and rapamycin up to 10 µM 30 min later, with the last receiving arabinose and rapamycin 30 min later. In total, samples were grown for 1 h, then diluted 10^{-5} and plated on L-agar with X-gal, thymidine and selective antibiotics.

Results

Strain construction

To investigate whether mutation of *topB* can increase the amount of precatenation in *E. coli's* genome to sufficient levels for detection using the ϕC31 attPB cassette excision assay, the *topB* knockout mutation, marked with a gentamycin resistance gene, was moved by P1 transduction into strains carrying the *attPB* Kan^{R} *lacI* cassette. Strains used had two different genetic backgrounds (DS941 or CP100) with the cassette located in the replication terminus region near the chromosomal dimer resolution locus *dif*, either without (DS941 *dif+*) or with (DS941 *Δdif*) the *dif* site deleted. CP100 is a *Rec+* strain with an intact *lacZ* gene, but with the lac repressor gene, *lacI*, deleted. Recombination by ϕC31 integrase deletes *lacI* in the *attPB* cassette, allowing expression of the chromosomal *lacZ* gene from the lac promoter, turning colonies blue on Xgal. DS941 is a *recF* mutant strain with a deletion in *lacZ,* so that this blue/white assay will not work. Precatenanes would be expected to be resolved last near the terminus, and in Δdif strains, no Xer recombination, which has been predicted to play a role in decatenation (Ip, 2003), can take place. Several gentamycin resistant colonies were obtained for each genotype, and PCR using primers flanking the *topB* gene confirmed that the *topB* knockout mutation was present in all.

Investigating precatenation in topB knockout mutants

To test whether precatenation could be detected in these strains, ϕC31 integrase was expressed from an arabinose inducible promoter on pZJ7m as described in Materials and Methods. Cells were treated with norfloxacin shortly after induction of integrase, to inhibit type II topoisomerases that would unlink any catenanes produced. Circular DNA was isolated and treated with a nicking endonuclease to remove supercoiling, and was separated on a high resolution agarose gel with the excised *attPB* cassette detected by Southern hybridisation (Fig 3).

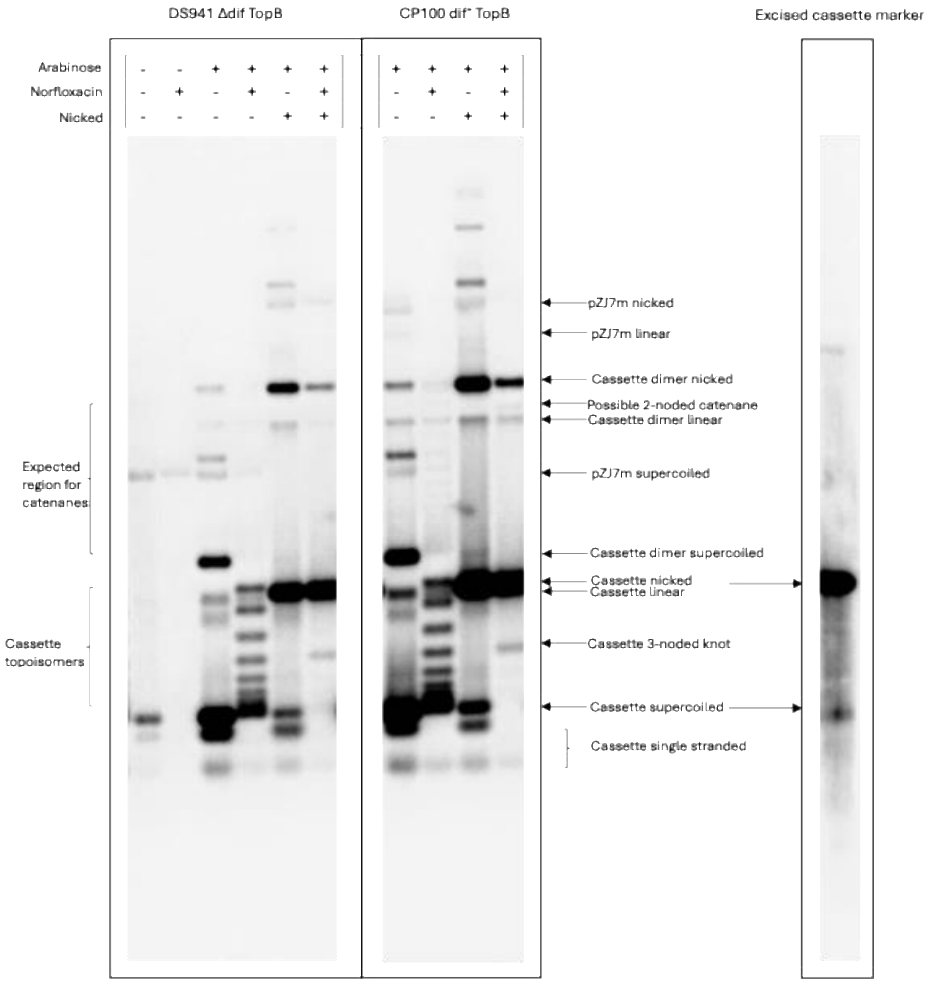


Fig 3: Southern blot to detect catenanes formed in *E. coli*'s chromosome. Excision by integrase of an *attPB* cassette on the chromosome was used to capture catenanes, if formed, with the excised cassette labelled in the figure. Results in strains DS941 *Δdif topB* and CP100 *dif*$^{+}$ *topB*, with integrase encoded by arabinose-inducible pZJ7m, are shown. A sample known to contain the nicked excised cassette, was used as a marker. The image is cropped, with lanes containing DS941 *dif*$^{+}$ *topB*, CP100 *Δdif topB*, and pZJ7m only, excluded. Results for other strains were similar to that shown here. Agarose gel was 1 % in TAE.

After induction by arabinose, the supercoiled excised cassette is seen. A cassette dimer resulting from inter-chromosomal recombination between the two newly replicated sister chromatids, is also observed as in previous studies by the lab (Tolmatcheva, 2019). Norfloxacin was added to inhibit type II topoisomerases, gyrase and TopoIV, further increasing time for which catenanes would be expected to persist, and created ladders of relaxed topoisomers for both the monomer cassette and cassette dimer. Less cassette dimer is seen for samples treated with norfloxacin (supported by analysis using ImageQuant software), as has also been previously observed, suggesting that norfloxacin reduces chances of recombination between newly replicated sister chromatids, perhaps as a result of altered supercoiling levels. Treatment with the nicking endonuclease, Nb.BbvcI, is seen to convert supercoiled cassette monomer and dimer to nicked forms.

Not observed in previous experiments, are the possible 3-noded knots of the cassette. This can perhaps be attributed to the absence of TopoIII in TopB strains, therefore with norfloxacin treatment, no topoisomerases able to resolve knots are active. Catenanes would be expected to run in the region between the supercoiled cassette dimer and the nicked cassette dimer, and their bands would not disappear with nicking. An unidentified band in this region has been labelled as a potential catenane, but further investigation is needed to confirm its identity.

Testing Split Integrase: transformation

Plasmids in Fig 2 were successfully constructed. Each integrase fragment (N-terminal amino acid 1-571 or C-terminal amino acid 572-605) was attached via a linker (L1) to a chemically inducible dimerization domain, either FKBP or FRB. Binding of rapamycin would make FRB and FKBP interact, therefore, the two integrase fragments would also come together to make a functional enzyme, as shown in Fig 4.

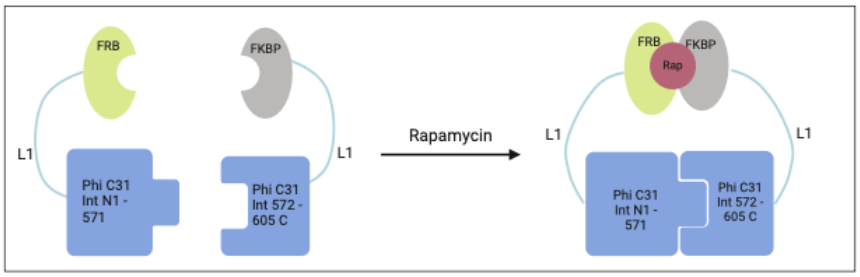


Fig 4: Schematic representation of split integrase. pCS3, pCS5 and pCS7 would encode the FKBP fragment (right hand of diagram), while pCS2, pCS4 and pCS6 would encode the FRB fragment (left hand side in diagram). Upon rapamycin binding to FRB and FKBP, integrase fragments would come together, creating a functional enzyme. (Created with BioRender.com)

CP100 *dif*$^{+}$ strains with the chromosomal *attPB* cassette were used to test recombination by split integrase, by means of a blue/ white colony colour assay.

Individual integrase fragments were unable to carry out *attPB* recombination on their own (Fig 5). CP100 *dif*$^{+}$ *topB* with both plasmids, grown on a plate without rapamycin, also produced white colonies only, with no background observed directly after transformation. When grown on rapamycin however, colonies were blue, indicating rapamycin could successfully dimerise the split integrase.

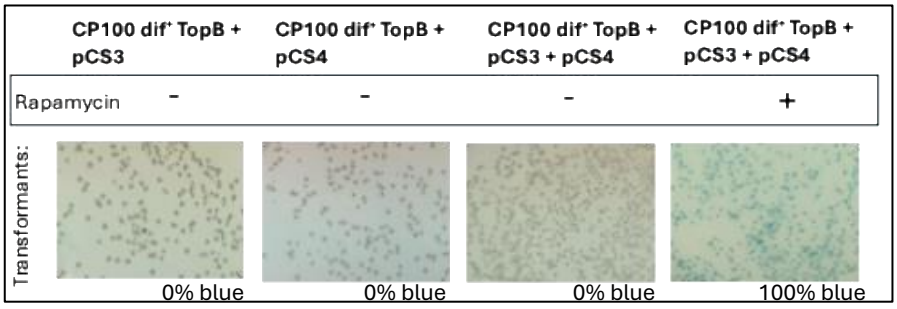


Fig 5: Results of transformations of CP100 *dif*$^{+}$ *topB* with pCS3 and PCS4. pCS3 encoded FKBP – L1 – ϕC31 Int 572-605 – NLS, while pCS4 encoded ϕC31 1-571 – L1 – FRB – NLS, both constitutively expressed. Transformants plated on L-agar with X-gal, thymidine, ampicillin and/ or chloramphenicol, with or without rapamycin, are shown. Recombination would result in excision of *LacI*, resulting in blue colonies.

Testing activity of Split-Integrase: time-course

A time-course was carried out to determine the rate at which rapamycin could induce the split integrase, with a low background of blue colonies observed amongst a majority of white, unrecombined colonies, before any addition of rapamycin. Upon addition of rapamycin, recombination was almost instantaneous, with all blue, recombined colonies observed from the 0 min timepoint.

Testing activity of Split-Integrase: rapamycin concentration

The effect of varying rapamycin concentration on recombination on the chromosome was investigated, yielding a clear trend of reducing recombination with decreasing rapamycin concentration (Fig 6). Close to 100 % of colonies were recombined down to a concentration of 1 µM rapamycin, and although blue colonies were observed without rapamycin, these were fewer than observed in the sample treated with the lowest concentration of rapamycin (0.01 µM).

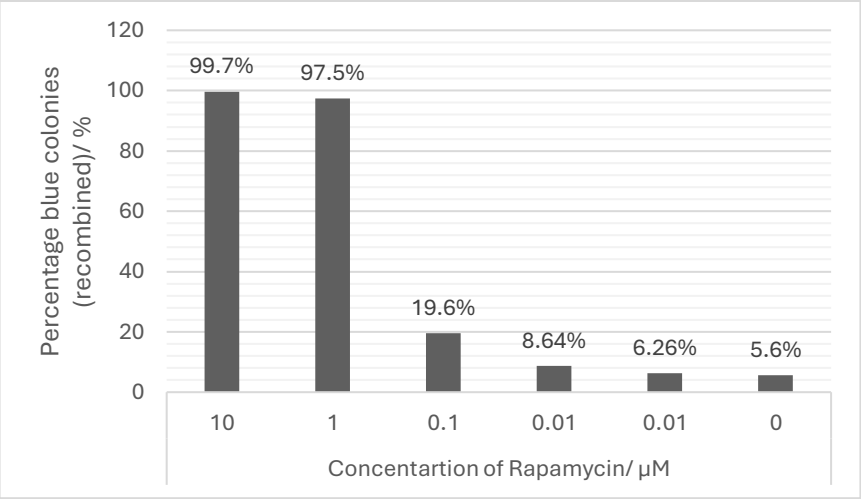


Fig 6: Recombination at the *attPB* cassette in CP100 *dif*⁺ *topB*, by integrase encoded by pCS3 (FKBP – L1 – ϕC31 Int 572-605 – NLS) and pCS4 (ϕC31 1-571 – L1 – FRB – NLS), with varying rapamycin concentrations. Liquid cultures were treated with rapamycin concentrations shown for 30 min, before plating a dilution on X-gal with thymidine, ampicillin and chloramphenicol. The experiment was only carried out once, so no error bars could be plotted.

Testing activity of Split-Integrase: arabinose inducible expression:

Testing induction of the split integrase, where one fragment was expressed from an arabinose-inducible promoter, revealed that addition of arabinose and rapamycin successfully induced

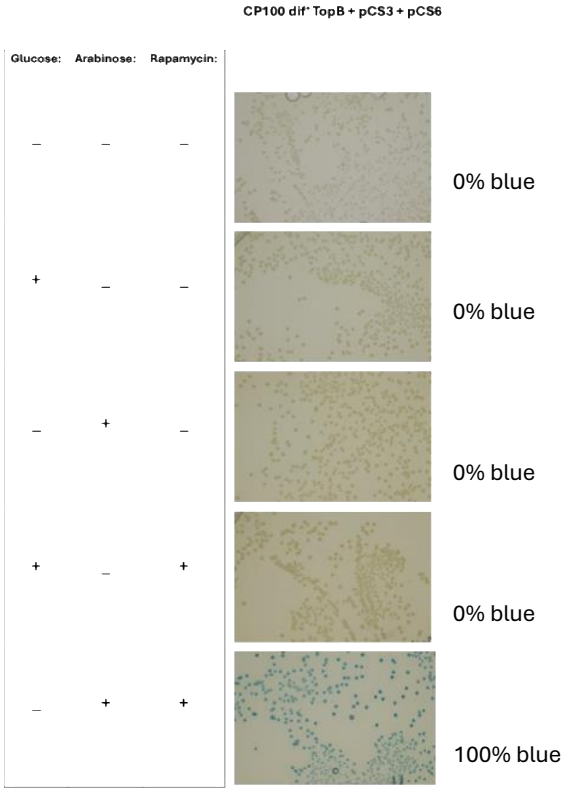


Fig 7: CP100 dif⁺ TopB transformed with pCS3 (FKBP – L1 – ϕC31 Int 572-605 – NLS) and pCS6 (ϕC31 1-571 – L1 – FRB – NLS, pBAD33 promoter). Glucose is the repressor of the pBAD33 promoter, while arabinose is the inducer. Cultures were grown for 1 h in total in various treatments, before plating on X-gal with thymidine, ampicillin and chloramphenicol. White colonies represent no recombination on the attP-attB cassette encoding LacI, while blue colonies represent recombination.

expression and dimerization, with all colonies recombined to become blue (Fig 7). No recombination was observed for other treatments, even for cells receiving arabinose, therefore expressing both integrase fragments. It can therefore be expected that having two 'checkpoints' for integrase activity removes most background recombination.

Discussion & Future Directions

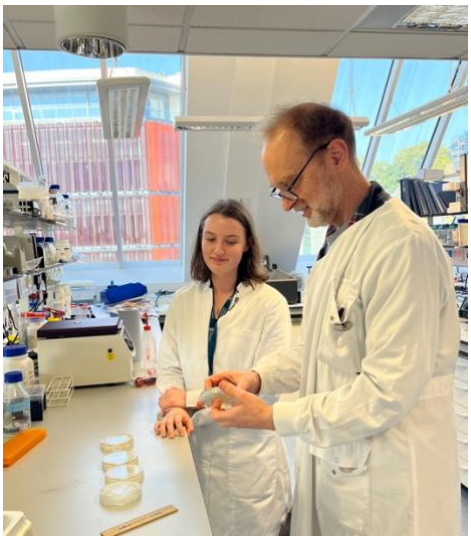
Although no catenanes could be detected with certainty, improvements have been made upon the assay, with a set of *topB* strains, possessing the *attPB,* cassette successfully constructed. These would be expected to increase persistence of precatenanes in the absence of TopoIII, improving chances of their detection in future experiments. To draw more reliable conclusions from the results of this investigation, samples could be treated with purified type II topoisomerase, TopoIV, which removes knotting and catenation. If the bands disappeared when viewed by gel electrophoresis, this would provide evidence of knotted or catenated species.

Blue/ white screens have suggested that the split integrase is able to recombine chromosomal *attPB* sites, but excision of the cassette could not be confirmed by standard agarose gel electrophoresis, staining with ethidium bromide. More sensitive detection methods, such as staining with SYBR Gold, or Southern hybridisation, can be used in future to confirm cassette excision. The split integrase allows rapid activation of recombination, independent of transcription and translation. This opens the possibility of investigating precatenation at precise timepoints in replication, by inducing integrase expression with addition of arabinose, at the same time as DNA replication initiation, and adding rapamycin at different time points to activate integrase and catenane capture by cassette excision.

As well as mapping precatenation over a time course, introducing more *attPB* cassettes throughout the chromosome would enable mapping of precatenation across the genome of *E. coli*. This would all contribute to a more complete view of DNA topology during bacterial DNA replication.

Transferable Skills Gained

Of course, spending over six weeks in a research laboratory improved my technical lab skills and introduced me to techniques I had not used before. Most importantly, it taught me to think more inquisitively and confirmed that spending my life in a research laboratory truly was my dream. I learned from planning, executing my own project, and record keeping in a lab book, improving on time management and organisational skills, which can be applied to many aspects of any future career. Furthermore, I gained experience working effectively as part of a research team, and clearly communicating my research and results.



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