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# Construction and evaluation of a bacterial fluorescent conjugation tracking system

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## 1. Abstract

Plasmids are small, circular, extrachromosomal genetic elements that replicate independently of the bacterial chromosome. Plasmids play a crucial role in the dissemination of antibiotic resistance genes through conjugation. Sub minimal concentrations of antibiotics have been shown to select for antibiotic resistance, but their effect on conjugation remains poorly understood. To study this, we constructed a Cre/lox system using a Cre-expressing plasmid and a chromosomal floxed BFP. We found that the system was not functioning as designed. Instead of expressing BFP upon conjugation, cells expressed BFP before conjugation and lost BFP expression upon recombination. We were able to locate the problem to the *loxP* site present after recombination, which interfered with the expression of BFP through two start codons present in the *loxP* spacer region. Both deletion and inversion of the *loxP* site present after recombination restored BFP expression to expected levels, providing strong evidence that the *loxP* sites were responsible for the opposite BFP expression observed.

**Keywords:** Antibiotic resistance, plasmids, conjugation, Cre/lox

## 2. Popular science summary

Antimicrobial resistance is a rapidly growing problem predicted to cause more deaths by 2050 than cancer is today. Antibiotics are a cornerstone of modern medicine and are ubiquitous in the healthcare setting. However, their ability to treat infections is rapidly dwindling due to bacterial resistance. Bacteria can become resistant to antibiotics through different mechanisms, with the uptake of plasmids being the most important.

Plasmids are small circular DNA molecules that can be transferred between bacterial cells. Plasmids often give bacteria an advantage by giving the bacterial cell carrying the plasmid resistance to one or more types of antibiotics. This makes plasmids bothersome in the clinic. They can quickly spread from cell to cell and sometimes even from patient to patient when antibiotics are present. In addition, as plasmids give the bacteria resistance to antibiotics, infections caused by bacteria carrying resistance plasmids are usually more challenging to treat – sometimes near impossible – and cause more severe infections.

Plasmids can jump from cell to cell through a process called conjugation. How antibiotics impact the frequency of conjugation is not well studied, which this study aims to address. We constructed a fluorescent Cre/lox system to study conjugation, which functions as an on-off switch. In the absence of conjugation, the system is silent, and no fluorescence is detected. However, in the presence of the plasmid, the Cre protein removes a piece of DNA that is preventing a fluorescent protein, Blue fluorescent protein (BFP), from being expressed. Removal of this piece of DNA turns the system on, and bacterial cells start expressing BFP, which we can detect in a machine as blue fluorescence. With this system, we can grow bacteria in the presence of different antibiotics at varying concentrations to see how the antibiotics impact the frequency of conjugation.

Initial trials with this system showed that it was not working as designed. Rather than being in the “off” state before conjugation, it was in the “on” state. It turned off upon conjugation, the opposite of how it was designed to work. The problem was located to a piece of DNA present in the on-off switch, the *loxP* site. This piece of DNA essentially tells the Cre protein what to remove; in this instance the piece of DNA stopping the expression of BFP. We show that both deletion and inversion of the *loxP* sequence puts the system in the “on” state after conjugation, which indicates that the *loxP* is causing the system not to function as expected.

While figuring out where the problem is located is a step in the right direction, there is still work to be done before the system is functional. The system needs to be redesigned from the beginning, with the *loxP* sites in the inverse direction from how they are presently oriented. This should make the system work as designed and allow us to track conjugation under different antibiotic conditions.

### 3. Introduction

#### 3.1 Antibiotic resistance

Antibiotics are arguably one of the most important discoveries of the 20<sup>th</sup> century. Since the discovery of penicillin by Sir Alexander Fleming in 1928, antibiotics have become a staple in modern medicine. However, even before the introduction of penicillin to the clinic in 1940, resistance in the form of penicillinases had already been described (1). This has been a constant theme in the history of antibiotics: new antibiotics are discovered only to find that there are already resistant populations out there, with clinical resistance developing shortly after introducing the new antibiotic to the clinic, or sometimes even before. The reasons for the increase in resistant populations are many, ranging from overuse, misuse, and over-prescription to extensive use in agriculture and the development of few new antibiotics (2,3). Any use of these wonderful drugs, in human medicine or in agriculture, will increase the frequency of resistance among human pathogens. This threatens the efficacy of antibiotics, and we will soon enter a post-antibiotic era, where we no longer have effective antibiotics with which to treat bacterial infections and the collapse of modern healthcare as we know it (4,5). We are heading towards a bleak future, with more people dying from diseases caused by resistant microbes than people are dying from cancer today, leading to trillions of dollars lost in world GDP (6).

There are many families of antibiotics, which can be grouped by their mechanism of action: (I) inhibitors of cell wall synthesis (e.g.,  $\beta$ -lactams), (II) inhibitors of protein synthesis (e.g., aminoglycosides), (III) inhibitors of DNA synthesis (e.g., fluoroquinolones), (IV) inhibitors of folic acid synthesis (e.g., sulphonamides), (V) inhibitors of RNA synthesis (e.g., rifampicin), and (VI) others (e.g., metronidazole). While their mechanisms of action may differ, they all have one thing in common: none have escaped the development of resistance mechanisms (5,7).

Just as the types of antibiotics are diverse, so are the resistance mechanisms that bacteria have developed. These can be divided into six groups: (I) prevention from reaching the target due to changes in cell permeability (e.g.,  $\beta$ -lactams), (II) active efflux (e.g., tetracyclines), (III) inactivation of the antimicrobial compound (e.g.,  $\beta$ -lactams and aminoglycosides), (IV) antimicrobial target alteration or protection (e.g., fluoroquinolones), (V) acquisition of alternative metabolic pathways (e.g., sulphonamides), and (VI) overproduction of antimicrobial target (e.g.,  $\beta$ -lactams and trimethoprim). Not all types of resistance mechanisms can be found for all kinds of antibiotics, but one or more are present for each group. Neither are the resistance mechanisms equal in the level of resistance they confer: group III-VI usually confers a high level of resistance, as is the case with the numerous  $\beta$ -lactamases, while group I confers a low level of resistance, and group II confers low-level intrinsic resistance to many classes of antibiotics (e.g.,  $\beta$ -lactams, aminoglycosides, and fluoroquinolones), and a high level of resistance towards tetracyclines, where the primary resistance mechanism is efflux by *tet*(A) and *tet*(B) (3,8–10).

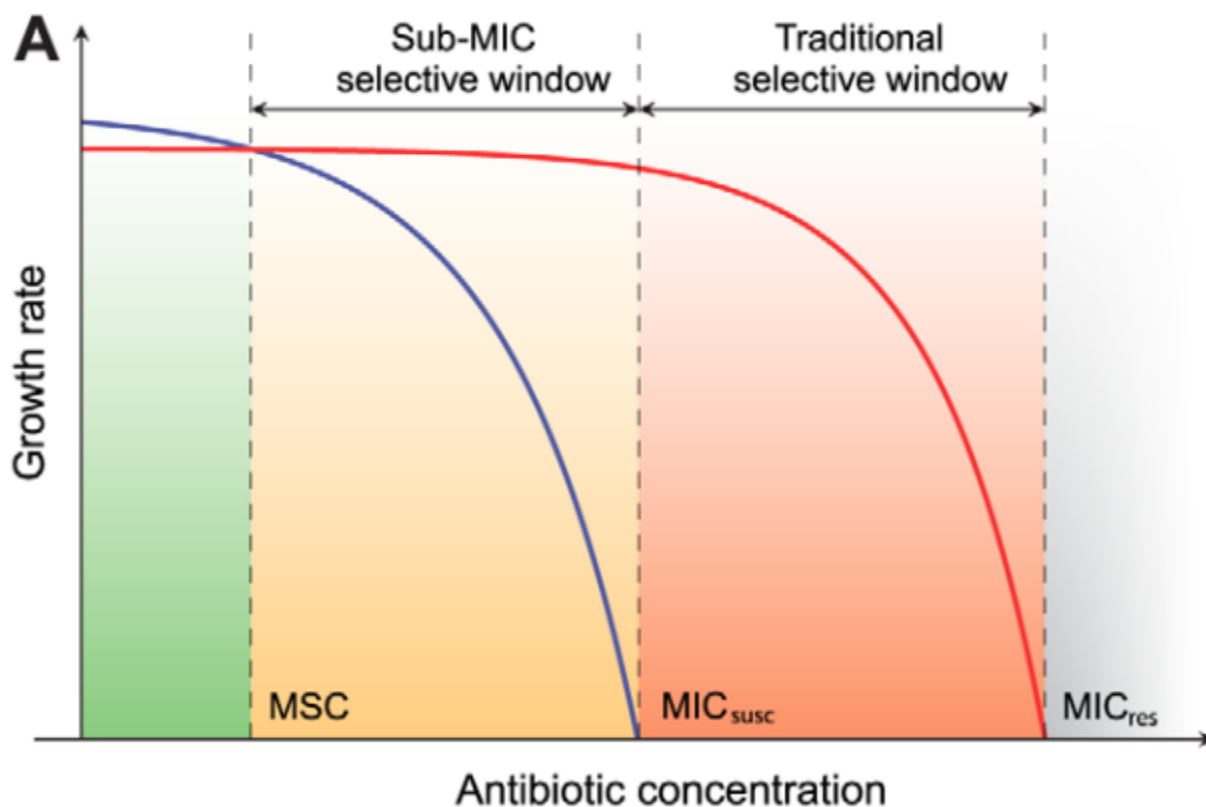
#### 3.2 Minimal inhibitory concentration

MIC, or the minimal inhibitory concentration, is defined as the lowest concentration of an antibiotic which prevents bacterial growth under specific *in vitro* conditions. It has been assumed that the selection of resistant subpopulations only occurs between the MIC of the susceptible WT population ( $MIC_{susc}$ ) and the MIC of the resistant subpopulation ( $MIC_{res}$ ). This hypothesis, the mutant selection window (**Figure 1**), has dominated the view on the selection of resistant mutants (11,12). However, more recent studies have shown the importance of sub-MIC concentrations of antibiotics. Sub-MIC concentrations have been shown to select both for *de novo* mutants (13,14), as well as for multi-drug resistance plasmids (15).

Resistance always comes at a price, generally measured as a reduction in growth rate compared to susceptible populations, termed fitness cost. Below the minimal selective concentration (MSC), any resistance comes at a high cost for the resistant cell, which is then outcompeted by faster-growing susceptible cells. At the MSC, any fitness cost associated with the resistance mechanism is balanced by an equally large fitness cost associated with the antibiotic pressure on the susceptible strain. Further increasing the antibiotic concentration tips the balance in favour of the resistant strains, as the fitness cost imposed on the resistant strain remains much the same, while the susceptible population suffers an ever-increasing

blow to their fitness. Thus, MSC is a function of fitness cost, and sub-MIC concentrations of antibiotics should enrich low fitness cost mutations (14,16).

These findings become clinically relevant when considering that the general strategy for treating infections is maintaining the highest possible concentration of antibiotics in the treated individual while minimising possible toxic effects. However, treatment often fails to reach a high enough concentration in all body compartments, where the antibiotic concentration falls in the sub-MIC category, and resistant subpopulations are thus selected. Furthermore, when considering that long-term exposure to sub-MIC levels of antibiotics is often desirable in agricultural practices to increase yield, the problem is further increased (16).



**Figure 1. Growth rate as a function of antibiotic concentration.** Green: Antibiotic pressure is low, and susceptible bacteria (blue line) grow faster than resistant mutants (red line). Orange: sub-MIC selective window where resistant mutants outcompete the susceptible strain. Red: Traditional selective window where the growth of the susceptible population is completely inhibited, and the resistant subpopulations suffers an ever-increasing reduction in growth rate. Adapted from Gullberg et al. (14).

### 3.3 Horizontal gene transfer and plasmids

There are two main ways by which resistance can develop in bacteria. Firstly, there is mutations in gene(s) associated with the mechanism of action of the antibiotic molecule. For any antibiotic-bacteria interaction, a subpopulation will develop resistance to the compound, leading to the survival of this subpopulation, while the susceptible population succumb to the effects of the antibiotic. Generally, resistance through mutation is slow, comes with a considerable fitness cost, and is only maintained in the population in the presence of the antibiotic (10). However, the fitness cost imposed by resistance mutations can often be alleviated by secondary fitness-compensating mutations (17).

Secondly, there is horizontal gene transfer (HGT), which allows bacteria to rapidly acquire resistance without going through the long and arduous process of acquiring multiple mutations. HGT comes in three forms: transduction by phages, transformation through uptake of foreign DNA from the environment, and conjugation of plasmids. Of these, conjugation plays the most crucial role clinically, as it allows the transfer of many resistance genes at once, giving a rapid development of clinical resistance to multiple antibiotics (18).

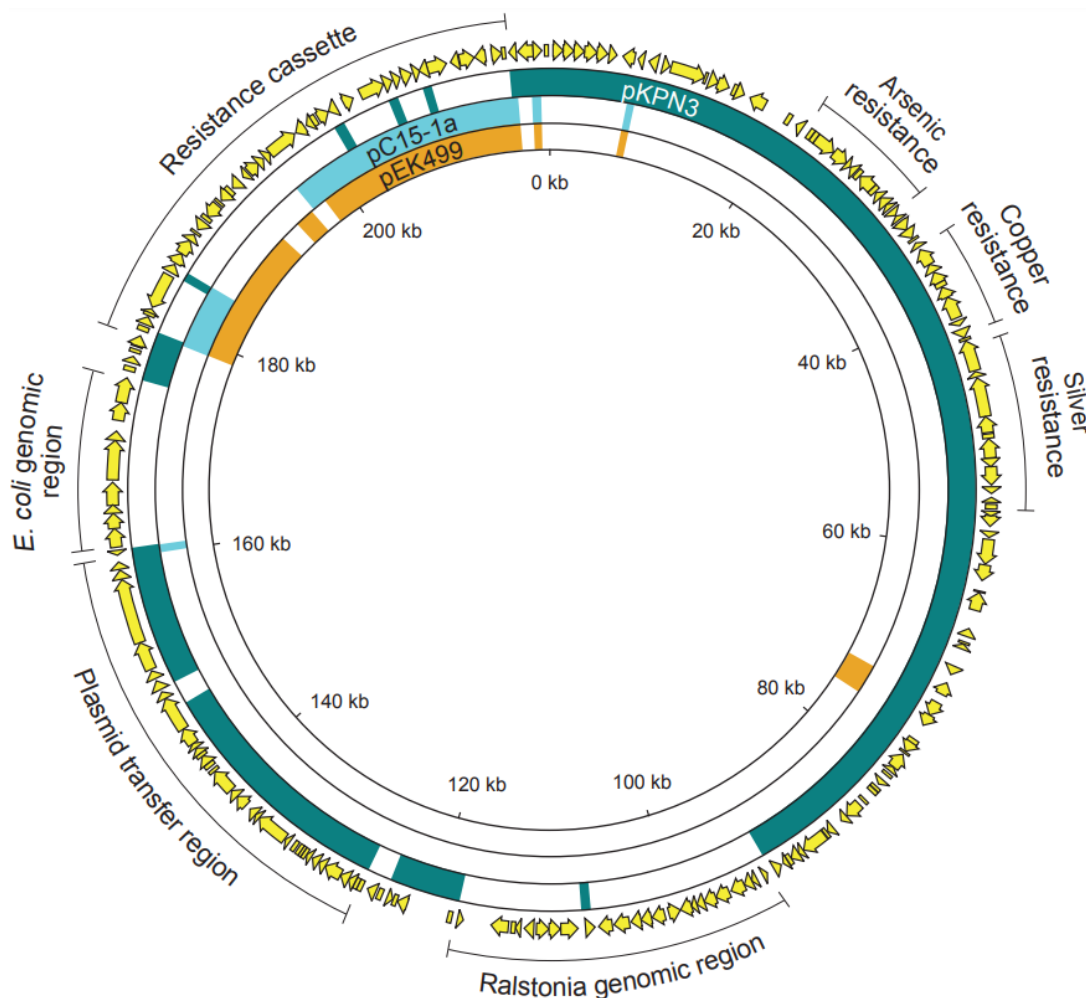
Plasmids are extrachromosomal genetic elements that can replicate independently of the chromosome. They are generally small, with an average size of 80 kbp (19), and often carry genes that are beneficial to the host bacterial cell under specific circumstances (e.g., antibiotic challenge) but are not essential for bacterial growth (e.g., central metabolism). Additionally, they encode the conjugative machinery responsible for the dissemination of the plasmid (20,21). Resistance to most classes of antibiotics can be found on plasmids, though genes conferring resistance to  $\beta$ -lactams, aminoglycosides, and tetracyclines are the most common (20). These are, coincidentally, the most used in human medicine and agriculture. In addition to antibiotic resistance genes, many plasmids also carry resistance genes to heavy metals (15,22–24). Selection and maintenance of the plasmid can happen by any one of the compounds to which it gives resistance, at concentrations well below the MIC of plasmid-free susceptible bacteria (15). These concentrations are readily found in polluted environments, sewage efflux, and antibiotic-treated humans and animals (14,25,26), suggesting that multi-drug resistance plasmids are maintained in the environment. Additionally, many plasmids also encode toxin-antitoxin systems, further promoting the stable maintenance of the plasmid in the population. These systems come in many forms but work on the same principle: a stable toxin and an unstable antitoxin. If the plasmid is stably inherited vertically, both toxin and antitoxin will be present, and the cell will survive. However, if the plasmid fails to partition itself, and one cell ends up losing the plasmid upon division, there is no longer any plasmid present to produce the antitoxin, leading to cell death by the toxin (27,28).

While even sub-MIC levels of antibiotics can select for antibiotic resistance genes, the effects of antibiotics on the frequency and dynamics of conjugation remains poorly understood. Antibiotics have been suggested to promote HGT (29), and the expression of a plasmid conjugation machinery has been shown to increase upon challenge with cefotaxime in an SOS-independent manner (30,31). However, previous research has often been conducted in a way that makes it impossible to distinguish between the effects an antibiotic might have on conjugation frequency and its effect on selection dynamics (32,33). Thus, the effects of antibiotics on conjugation remains unclear.

Inhibiting conjugation has been suggested as a way to prevent antibiotic resistance and extend the lifetime of our antibiotics (34), underscoring the importance of unambiguously discerning the effects antibiotics might have on conjugation and dissemination of plasmids.

### 3.4 pUUh239.2

Between 2005 and 2010, a large nosocomial outbreak of extended-spectrum  $\beta$ -lactamase (ESBL) producing *Klebsiella pneumoniae* occurred at the Uppsala University Hospital, Uppsala, Sweden. During the outbreak, around 300 patients were either infected or colonised, mostly elderly patients with a median age of 78. This outbreak was caused by a clonal strain of *K. pneumoniae* carrying a large (220 kbp) IncFII plasmid, the pUUh239.2 plasmid (**Figure 1**). This multi-resistance plasmid contains genes encoding resistance to  $\beta$ -lactams (*bla*<sub>TEM-1</sub> and *bla*<sub>OXA-1</sub>, and the ESBL *bla*<sub>CTX-M-15</sub>), tetracycline (*tet*(A) and *tetR*), aminoglycosides (*aadA2*) macrolides [*mphR*(A), *mrx*, and *mph*(A)], trimethoprim (*dhfrXII*), sulphonamides (*sulI*), and aminoglycosides/fluoroquinolones (*aac*(6')-Ib-cr). In addition to the extensive antibiotic resistance cassette, the plasmid also carries genes conveying resistance to arsenic, copper, and silver. The plasmid was also found in unrelated *E. coli* present in the gut of infected patients, indicating a horizontal spread of pUUh239.2 across species barriers, highlighting the importance of a proper understanding of conjugation dynamics under antimicrobial challenge (35,36).



**Figure 2. The pUUH239.2 plasmid.**

### 3.5 Cre/*loxP* recombination

The Cre/*loxP* is a powerful genetic tool, allowing site-specific manipulations of any genome. It has been extensively used in mouse genetics (37,38) and bacterial genome editing (39). The system consists of two parts: (I) The Cre recombinase (Causes recombination) of bacteriophage P1, and (II) the 34 bp recognition sites, named *loxP* (40). The Cre recombinase is a site-specific recombinase, which catalyses the recombination between two *loxP* sites. The orientation of the *loxP* sites decides the outcome of recombination: cis *loxP* sites with the same orientation cause deletion and *loxP* sites with opposite orientations causes inversion. In contrast, trans *loxP* sites allow for translocation between two DNA molecules. The *loxP* sites are 34 bp (5' - ATAACCTTCGTATAATGTAATGCTATACGAAGTTAT - 3'), consisting of an eight bp spacer region flanked by two 13 bp palindromic sequences. Recombination occurs within the spacer region, creating a hybrid of the two *loxP* sites while removing the other (41). Any DNA sequence flanked by two *loxP* sites is said to be floxed. The Cre/*loxP* system offers a powerful way to study conjugation. By placing a reporter gene which is being silenced by floxed terminators on the chromosome of a recipient strain and introducing the Cre recombinase on a conjugative plasmid, conjugation could be effectively tracked. Before conjugation the floxed reporter gene remains silent. Upon successful conjugation of the plasmid carrying the Cre recombinase, the reporter would turn on, and each conjugation event could thus be tracked.

### Aim

The aim of this study was (I) to construct a bacterial conjugation tracking system utilising the Cre/*loxP* system, and (II) to evaluate the system to study the effects sub-MIC levels of antibiotics have on conjugation.

## 4. Materials and methods

### 4.1 Strain construction

Most strains used in this study were derived from *Escherichia coli* MG1655 or *Salmonella enterica* serovar Typhimurium LT2 and can be found in **Table 1**. Bacteria were grown in Luria-Bertani broth (LB) or on Luria-Bertani agar (LA; Sigma-Aldrich, MO, USA) supplemented with antibiotics as appropriate for selection or maintenance of plasmids at the following concentrations: ampicillin 100 mg/L; chloramphenicol 12.5 mg/L; kanamycin 50 mg/L; spectinomycin 50 mg/L; and tetracycline 10 mg/L. Sigma-Aldrich supplied all antibiotics.

**Table 1.** Bacterial strains

Strain	Genotype	Comment
DA5438	<i>E. coli</i> MG1655 WT	
DA21802	<i>E. coli</i> NEB 5-alpha / pSB1A2-J61047	Part BBa_J61047 from the Registry of Standard Biological parts
DA24100	<i>E. coli</i> MG1655 / pSIM5-tet	Lambda red plasmid.
DA25015	<i>E. coli</i> MG1655 / pSIM5-cam / pJNEG01 (miniF, YFP-bla)	Lambda red plasmid. YFP on conjugative plasmid pJNEG01
DA27284	<i>E. coli</i> MG1655 <i>galK</i> ::cat-J23110-mTagBFP	Synthetic promoter J23110 followed by floxed terminator and mTagBFP
DA27758	<i>E. coli</i> DH5-alpha/ pSB4S15-J23116-Cre	Cre recombinase and <i>aadA</i> plasmid.
DA28200	<i>E. coli</i> MG1655 <i>galK</i> ::FRTscar-J23101-SYFP2	Constitutively active chromosomal YFP
DA28202	<i>E. coli</i> MG1655 <i>galK</i> ::FRTscar-J23101-mTagBFP2	Constitutively active chromosomal BFP
DA28851	<i>S. Typhimurium</i> LT2 <i>galK</i> ::kan-J23101-mTagBFP2	Synthetic promoter J23101 followed by floxed terminator and mTagBFP2
DA32004	<i>E. coli</i> MG1655 <i>galK</i> ::kan-J23101-mTagBFP2	BFP switch from DA28551 inserted in <i>galK</i>
DA71922	<i>E. coli</i> MG1655 / pSC101	pSC101 plasmid for reconstruction of Cre/YFP construct

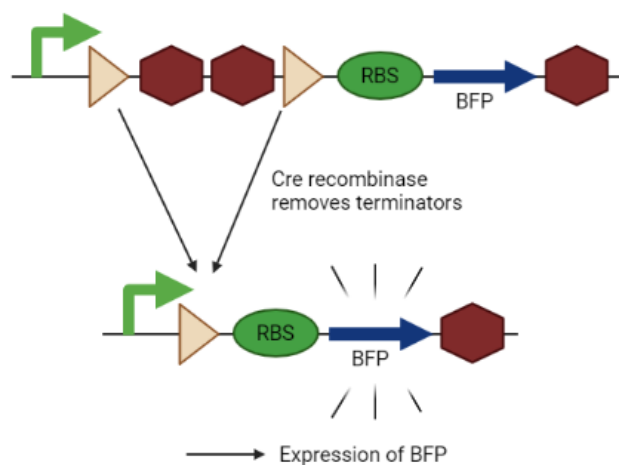
### 4.2 Polymerase chain reaction

All PCR reactions were performed using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., MA, USA) on a C100 Touch™ Thermal Cycler (Bio-Rad, CA, USA). Cycling conditions can be found in **Supplementary Tables 1 and 2**. Primers used can be found in **Supplementary Table 3**. PCR products were stained with GelRed® Nucleic Acid Gel Stain (Biotium, CA, USA), separated on a 1% agarose gel and visualised under UV light. The GeneRuler 1 kb DNA Ladder (Thermo) was used to determine size. GeneJET Gel Extraction Kit (Thermo) was used to purify PCR products, following the manufacturer's instructions, either from gel or directly from the PCR reaction. Purified PCR products were stored at -20°C.

### 4.3 BFP lox-switch

The mTagBFP2 cassette was amplified from DA28851 using the primers pEL\_galK\_Eco\_F and ColiGalKYFPLinR and inserted into *galK* of DA24100 using lambda red recombineering as described below, with the resulting strain being named DA32004. **Figure 3** contains a schematic overview of DA32004. The BFP lox-switch consists of the J23101 promoter (BBa\_J23101, Registry of Standard Biological Parts, (42)) with its associated RBS (BBa\_B0034), which can drive the expression of mTagBFP2. However, two terminators stop any expression: the T7 terminator (BBa\_B0012) and lambda T1 terminator (BBa\_K864601), both in the forward direction. The two terminators are floxed, which allows

for their removal upon recombination by the Cre recombinase, with the subsequent expression of mTagBFP2.



**Figure 3.** BFP lox-switch in DA32004. Two terminators are flanked by *loxP* sites, stopping the expression of BFP. The terminators are removed upon recombination, and BFP is expressed. Made using Biorender.com.

#### 4.4 Lambda red recombineering

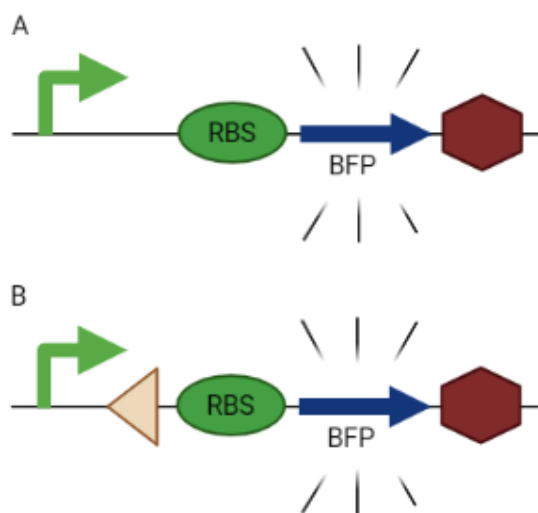
Lambda red recombineering was performed as previously described by Datsenko and Wanner (43). DA24100 was grown in liquid LA culture overnight at 30°C supplemented with 10 mg/L tetracycline and 200 r.p.m. shaking. 500 µL overnight culture was added to 50 mL fresh LA with 10 mg/L tetracycline and grown at 30°C and 200 r.p.m. shaking until  $OD_{600} \approx 0.2-0.3$ . Red expression was induced by placing cultures in a 42°C water bath with 110 r.p.m. shaking for 15 minutes, after which cells were placed on ice for 10 minutes to cool down. Cells were washed three times in 15 mL 10% glycerol (VWR International, PA, USA) and centrifuged at 4200 x g for 10 minutes to prepare them for electroporation. 40 µL of cells and up to 200 ng purified PCR product was mixed and transferred to 1 mm gap electroporation cuvettes (VWR). Electroporation was done at 2.5 kV, 200Ω, and 25µF in a Gene Pulser Xcell electroporator (Bio-Rad). Cells were transferred to 1 mL SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 2 mM MgSO<sub>4</sub>, and 20 mM glucose) pre-warmed to 37°C and were allowed to recover for at least 3 h or overnight at 30°C and 200 r.p.m. shaking before plating on selective media. Prepared DNA was stored at -20°C and thawed on ice in preparation for electroporation.

#### 4.5 Plasmid transformation

Plasmid transformations were performed following the same protocol described for lambda red above, except that cells were grown at 37°C to an  $OD_{600} \approx 0.5-0.6$  with no 42°C induction step. Plasmids were used rather than PCR products for electroporation, and selection post electroporation was done using appropriate antibiotics.

#### 4.6 Deletion/inversion of *loxP* site post recombination

The *loxP* site present after recombination was either deleted or inverted using an assembly PCR with DA32004 as a template. Four PCR reactions were performed using the primer pairs: (1) galK\_scrn\_F and Lox\_deletion\_R or Lox\_inversion\_R, and (2) Lox\_deletion\_F or Lox\_inversion\_F and galK\_scrn\_R. Cycling conditions can be found in **Supplementary Table 1**. The products from (1) and (2) were used as a template for a PCR using the primers galK\_scrn\_F and ColiGalK\_SF. Cycling conditions can be found in **Supplementary Table 2**. The product was used for lambda red insertion into DA24100 as previously described. An overview of the resulting strains can be found in **Figure 4**.



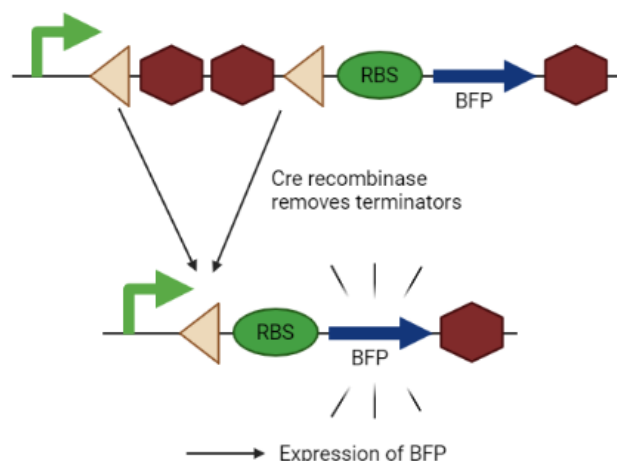
**Figure 4.** (A) *loxP* site deleted after recombination, and (B) *loxP* site inverted after recombination using assembly PCR. Made using Biorender.com.

#### 4.7 Inversion BFP lox-switch

Both *loxP* sites were inverted using multiple assembly PCR reactions (**Figure 5**). First, a PCR was performed on DA32004 using the primers ColiGalK\_SR and galK\_scrn\_R. Cycling conditions were as described in **Supplementary Table 1**. This produced a PCR product of the entire BFP switch region, which was then used as a template for further PCR reactions to minimise unwanted primer binding. Four PCR reactions were set up using the following primer pairs: (1) ColiGalK\_SR and Lox\_inversion\_R, (2) BFP\_Inv\_F\_Term and BFP\_Inv\_R1\_Term, (3) Lox\_inversion\_F and GalK\_scrn\_R, and (4) BFP\_Inv\_F\_Term and BFP\_Inv\_R1\_AP. Cycling conditions can be found in **Supplementary Table 1**. The purified products from reactions (1) and (2) were used as a template for reaction (5) using the primers ColiGalK\_SR and BFP\_Inv\_R1\_Term, and products from (1) and (4) were used as templates for reaction (6) using the primers ColiGalK\_SR and BFP\_Inv\_R1\_AP. Cycling conditions were as described in **Supplementary Table 2**. The product from reaction (5) was used as a template for reaction (7) using the primers ColiGalK\_SR and BFP\_Inv\_R2\_term, and the product from reaction (6) was used as a template for reaction (8) using the primers ColiGalK\_SR and BFP\_Inv\_R2\_AP. Cycling conditions can be found in **Supplementary Table 1**. The products from reactions (3) and (7) were used as templates for reaction (9), and products from reactions (3) and (8) were used as templates for reaction (10), using the primers galK\_scrn\_F and ColiGalK\_SF. Cycling conditions can be found in **Supplementary Table 2**. Products were purified from gels using GeneJET Gel Extraction Kit (Thermo) to remove unwanted products and stored at -20°C. Products (9) and (10) were used for lambda red insertion into DA24100 as previously described. Inserts were screened using primers OL\_galK\_scrn\_F and OL\_galK\_scrn\_R. Cycling conditions can be found in **Supplementary Table 1**.

#### 4.8 Plasmid DNA preparations

Plasmids were purified using the NucleoBond® Xtra Midi kit (Macherey-Nagel, Düren, Germany), using the low copy plasmid purification protocol, following the manufacturer's instructions. Plasmid concentration was determined using a NanoDrop 1000 spectrophotometer (Saveen Werner, Malmö, Sweden), and the purified plasmids were stored at -20°C.



**Figure 5.** Inversion of both *loxP* sites using an assembly PCR. Made using Biorender.com.

#### 4.9 Cre recombinase plasmid

A plasmid preparation was done on strain DA27758, and the plasmid was digested using MluI following the manufacturer's instructions (Thermo; **Supplementary Table 4**). The digested plasmid was used as a template for a PCR reaction with the primers pEL\_pJNEG\_F and pEL\_pJNEG\_R. The purified PCR product was used for lambda red insertion into the pJNEG01 plasmid of DA25015 as previously described. Successful insertion was confirmed by two PCRs: (1) using the primers Ver\_YFP\_F and Ver\_YFP\_R, and (2) using the primers pEL\_pJNEG\_R and Ver\_YFP\_R. Cycling conditions can be found in **Supplementary Table 2**. Transformants were selected on 50 µg/mL spectinomycin. Plasmids were prepared from transformants as described above and transformed into appropriate *loxP* strains to introduce Cre recombinase.

#### 4.10 Fluorescence analysis

Cells were grown overnight at 37°C and 200 r.p.m. shaking and appropriate antibiotics. 1 µL of overnight culture was diluted in 100 µL PBS in 96-well plates or single-use tubes and was allowed to develop fluorescence for 30 minutes at room temperature before analysis using a MACSQuant®VYB (Miltenyi Biotec, Bergisch Gladbach, Germany).

#### 4.11 Sequencing

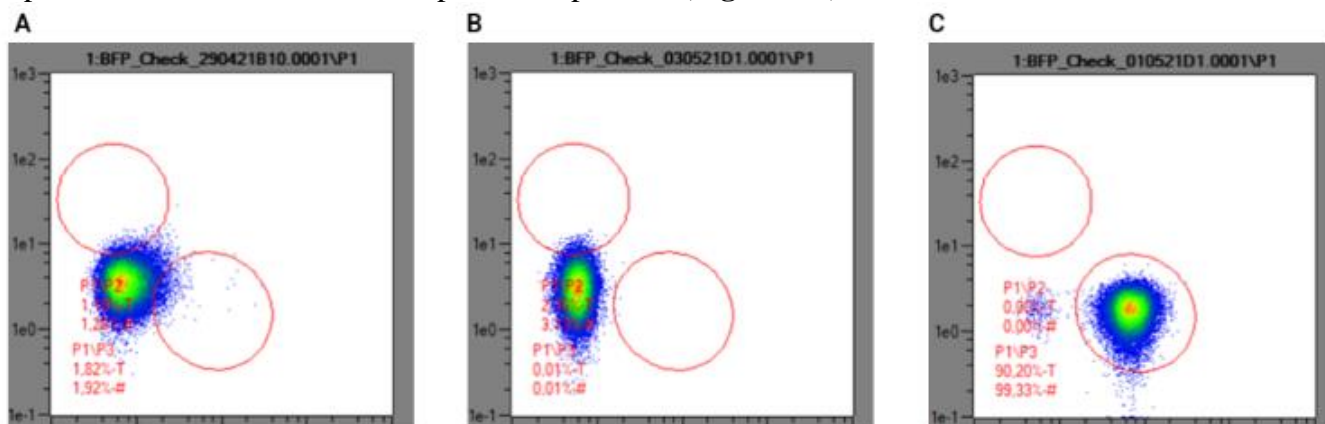
Purified DNA was used as a template for Mix2Seq Sanger sequencing (Eurofins Genomics, Ebersberg, Germany), following the manufacturer's instructions. BFP switch regions were amplified by PCR using the primers OL\_galK\_scrn\_F and OL\_galK\_scrn\_R. Cycling conditions can be found in **Supplementary Table 1**. Products were purified using GeneJET Gel Extraction Kit (Thermo), and DNA concentration was measured using Qubit™ dsDNA BR Assay Kit on a Qubit™ 2.0 Fluorometer (Invitrogen, MA, USA). 10 ng/µL purified DNA was mixed with 2 µL [10µM] of each primer and sent for sequencing. The BFP switch was sequenced using five primers: ColiGalK\_SR, VF\_2, Lox\_inversion\_F, BFP\_ampl\_R, and galK\_scrn\_R.

### 5. Results

#### 5.1 pJNEG01 Cre plasmid does not express YFP

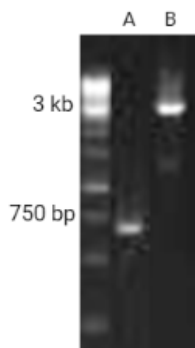
The plasmid construct consisted of three parts: The Cre recombinase, a constitutively active YFP, and a resistance gene. The entire construct could then be transferred to a plasmid of interest, i.e. pUUH239.2. The Cre recombinase and spectinomycin resistance gene (*aadA*) was amplified from DA27758 and inserted into the pJNEG01 plasmid of DA25015 through lambda red recombineering. The finished plasmid was transformed into DA32004, and fluorescence was measured using MACS. Transformants were grown on

spectinomycin, selecting for the insertion of the amplified fragment into pJNEG01. Surprisingly, no YFP expression was observed from the pJNEG01 plasmid (**Figure 6A**).



**Figure 6. DA32004 does not express YFP upon transformation with pJNEG01 plasmid but undergoes recombination.** 1  $\mu$ L overnight culture was diluted in 100  $\mu$ L PBS and allowed to develop fluorescence for 30 minutes at room temperature before they were analysed using MACS. 50,000 cells were counted. Y-axis shows BFP expression, and X-axis shows YFP expression. (A) DA32004 transformed with finished pJNEG01 plasmid, carrying a constitutively active YFP, in addition to Cre recombinase and spectinomycin resistance (*aadA*). (B) DA32004 transformed with Cre plasmid without YFP (DA21802). (C) Expected YFP fluorescence (DA28200).

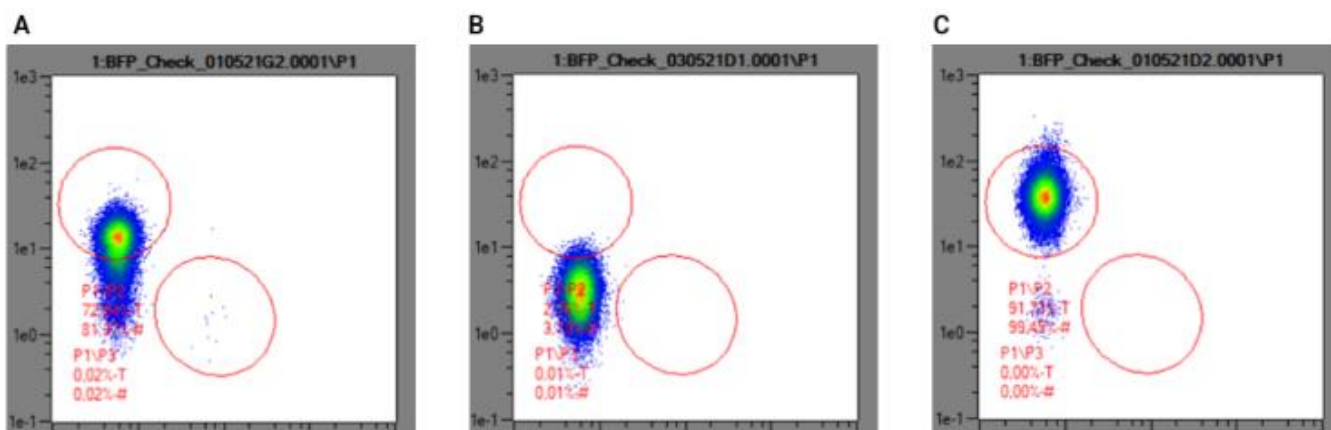
PCR confirmed that the insertion took place as expected. If the entire YFP gene is present, a band would be expected at 700 bp, which was also observed (**Figure 7A**). Thus, the YFP gene seems undamaged by the insertion of the Cre recombinase and spectinomycin resistance gene. Verification of the entire construct on the pJNEG01 plasmid yielded the expected 3.2 kb band (**Figure 7B**), but YFP is nevertheless not expressed to any significant degree.



**Figure 7. PCR confirms the pJNEG01 construct.** (A) Verification of YFP on pJNEG01. Product expected at 700 bp. (B) Verification of entire pJNEG01 construct. Product expected at 3.2 kb.

## 5.2 DA32004 displays BFP expression opposite of design

DA32004 was designed so that BFP should not be expressed before recombination due to the two terminators. Upon recombination by the Cre recombinase, the terminators are supposed to be removed, and BFP can be expressed. To verify the construct, the fluorescence of DA32004 was analysed using MACS, with and without the addition of the Cre-expression plasmid. The main DA32004 population of cells were clearly expressing BFP before recombination (**Figure 8A**) and lost BFP expression upon recombination (**Figure 8B**).



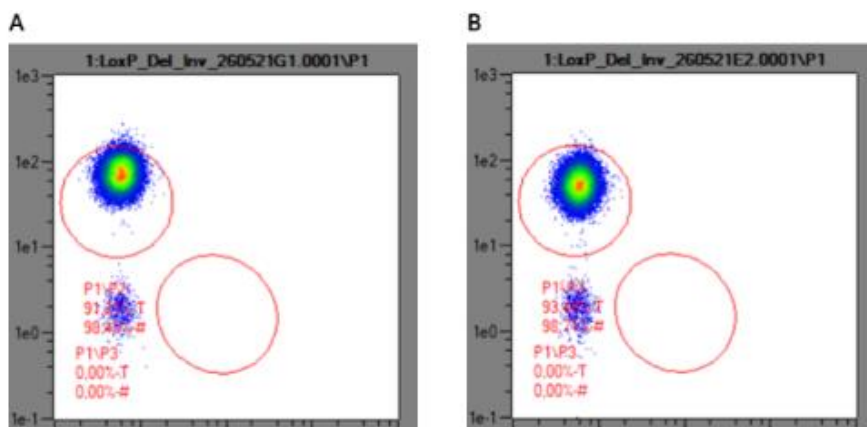
**Figure 8. DA32004 loses BFP expression after recombination.** (A) DA32004 before recombination. Terminators are present, but BFP is still being expressed. (B) DA32004 after transformation with Cre-expression plasmid. Terminators are removed, while BFP expression is lost. (C) DA28202 with constitutively active BFP. The expected BFP expression level after recombination of DA32004.

This pattern of BFP expression was the opposite of what was expected from the design of the construct. Both strains were sent for sequencing, which confirmed that the sequences were as expected. The *loxP* sites of DA32004 were located in the correct places around the two terminators, with the correct orientations, and contained no mutations that could explain the leaky expression seen in **Figure 8A**. Additionally, the subsequent strain had the correct DNA sequence, with both terminators removed after recombination. The *loxP* site present after recombination was the only piece of DNA present in the recombined DA32004 strain compared to DA28202 (BFP before introducing the lox-switch), which displayed normal BFP expression (**Figure 8C**). It was thus hypothesised that there was something in the *loxP* site which caused the strange BFP expression. Further analysis of the sequence revealed two start codons present in the spacer region of the *loxP* site, marked in red: 5' - **ATG**T**ATG**C - 3'. The start codons may interfere with the expression of BFP.

### 5.3 Deletion or inversion of *loxP* site after recombination restores BFP expression

To test whether the start codons were responsible for the expression patterns observed, the *loxP* site was either deleted or inverted using PCR on DA32004 cells which had undergone recombination (**Figure 8B**). Deletion of the *loxP* site should restore the sequence to that of DA28202, and inverting it would make the start codons in the spacer region face the other direction. Both conditions should produce BFP expression levels comparable to those of DA28202.

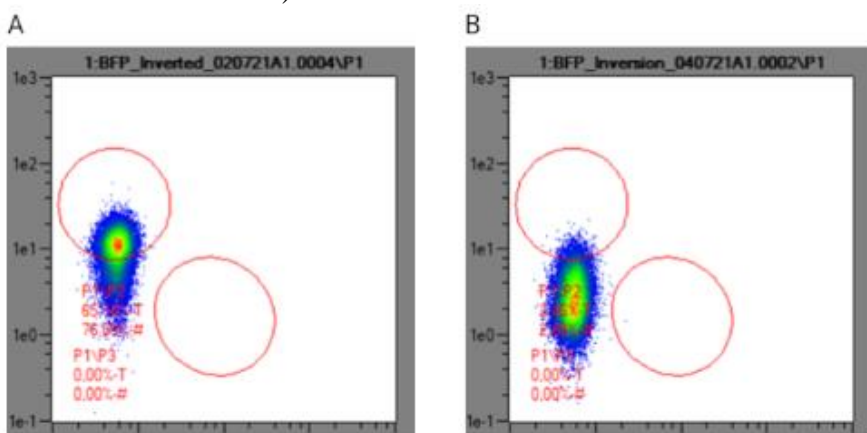
As seen in **Figure 9A**, deletion of the *loxP* site restores the expression to comparable levels as in strain DA28202. This provides strong evidence that the *loxP* site is somehow causing the disturbed BFP expression seen in DA32004. However, there will always be a *loxP* site present after recombination, which is why it was also inverted to remove the putative interference while keeping the lox-switch functional. Inversion of the *loxP* site also restored BFP expression to levels comparable to DA28202 (**Figure 9B**), providing further evidence that the *loxP* site contains something directional, presumably one or both start codons, responsible for causing the BFP expression observed in DA32004.



**Figure 9. BFP expression was restored after deletion or inversion of *loxP*.** DA32004 was transformed with the Cre-expression plasmid from DA21802. Transformants underwent recombination, removing all but one *loxP* site, which was then either deleted (A) or inverted (B) using assembly PCR. Both strains show BFP expression levels comparable to DA28202.

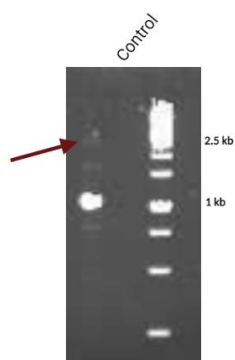
#### 5.4 Construction of the BFP switch with both *loxP* sites inverted

To confirm that the BFP switch now worked as expected, both *loxP* sites of DA32004 were inverted using multiple assembly PCR reactions. The final PCR products were used for lambda red recombineering into DA24100, resulting in a strain identical to DA32004, except for the two inverted *loxP* sites. A Cre-expression plasmid was transformed into the strain, and the recombined and non-recombined strains were analysed by MACS. Unfortunately, the strains showed identical BFP expression to DA32004 (**Figure 10**). Sequencing revealed that these new constructs had identical sequences to DA32004 (**Supplementary Notes 1**), either indicating that the assembly PCR reactions failed or that there was a template (i.e. BFP switch from DA32004) contamination of at least one of the PCR reactions after purification.



**Figure 10. Assembly PCRs failed to invert *loxP* sites.** *loxP* sites were inverted using multiple assembly PCR reactions using DA32004 as a template. The finished construct was used for lambda red insertion into DA24100. The resulting strains show identical BFP expression to DA32004 before (A) and after (B) recombination.

Most PCR reactions were purified from gels to remove unwanted products. However, reaction 3 using the primers *Lox\_inversion\_F* and *GalK\_scrn\_R* was not and might thus have introduced DA32004 template DNA into the final reaction to combine the inverted *loxP* fragments (**Figure 11**). This template DNA would then be preferentially amplified in favour of the inversion fragments. This would explain why seemingly nothing happened during the assembly PCR.



**Figure 11. PCR reaction with possible template contamination.** Product expected at 1 kb - possible template contamination at 2.5 kb (Red arrow).

## 6. Discussion

This study aimed to construct and evaluate a conjugation tracking system. The imagined system consisted of two parts: (1) a conjugative plasmid carrying a constitutively active YFP and the Cre recombinase, and (2) a floxed chromosomal BFP. While neither system is currently functional, the ideas still show promise but require additional work before implementation.

### 6.1 Cre/YFP plasmid

The idea was to build the Cre/YFP cassette on a construction plasmid, pJNEG01, where the system could be modified before transferring the cassette to any plasmid of interest (e.g., pUUH239.2). There were a few problems with this system. Firstly, the pJNEG01 is quite large at ~57 kb, which caused problems during the plasmid transformation. As the plasmid was only used for the construction of the cassette and not for any actual conjugation experiments, transformation was used in favour of conjugation as a means of introducing the plasmid into relevant cells, as transformation is less labour-intensive. However, due to its large size, transformation proved difficult, and the very low frequencies observed makes the plasmid cumbersome to work with. The system was later repurposed for the pSC101 plasmid (44), a significantly smaller plasmid that gave transformation frequencies  $>10^6$ -fold higher than pJNEG01. As the plasmid was never meant to be used in any conjugation experiments, only for the cassette construction, more consideration should have been given to the size of the plasmid.

Secondly, YFP was placed under an IPTG-inducible promoter, PLlacO1 (BBa\_R0011), rather than a constitutively active promoter. Although the entry for pLlacO1 on the Registry of Standard Biological Parts (parts.igem.org) previously claimed that the promoter was constitutively active, it has since been edited. A constitutively active promoter is crucial for our design. Work is underway to replace the PLlacO1 promoter with five different promoters from the J23100 family of promoters to allow fine-tuning of the YFP expression levels.

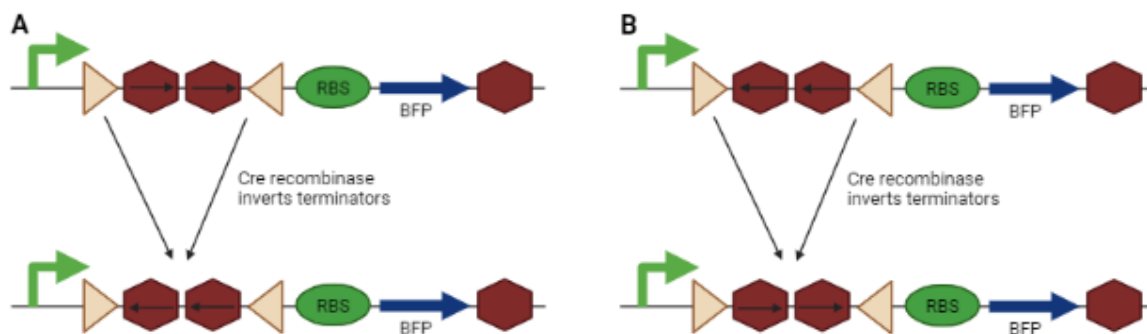
Lastly, the antibiotic resistance gene (*aadA*) needs to be replaced with something more suitable for pUUH239.2, such as the *cat* gene for chloramphenicol resistance, as pUUH239.2 already carries resistance genes to spectinomycin, making screening of the cassette impossible.

### 6.2 BFP switch

The BFP switch was designed so that the BFP would be silent before recombination and only turned on upon conjugation of the Cre/YFP plasmid. However, the first trials gave the opposite results; BFP was expressed before recombination, and all expression was lost after recombination (**Figure 8**). There are a few explanations as to why the expected BFP expression is not observed.

Assuming that the *loxP* sites were incorrectly incorporated in the construct and are facing opposite directions rather than the same direction, recombination would invert the terminators, as seen in **Figure 12A**. However, this arrangement fails to explain the drop in BFP expression. If anything, this should lead to a higher BFP expression as the T7 terminator is a promoter in the reverse direction, which it would now be facing. If the terminators were facing the wrong direction, as seen in **Figure 12B**, and the *loxP* sites

were facing opposite directions, the observed BFP expression could be explained. Before recombination, the terminators are facing the wrong way, and BFP can be expressed. Upon recombination, the terminators would be inverted and face the correct way to stop BFP expression, which was observed.



**Figure 12. Hypothetical BFP lox-switches.** (A) *loxP* sites have been inverted, and (B) both *loxP* sites and terminators have been inverted.

However, sequencing revealed that the construct had the expected DNA sequence and that the terminators were removed upon recombination. There are then two options remaining: either there is something in the *loxP* site left after recombination disturbing BFP expression or the promoter cannot drive BFP expression, and the expression of BFP seen before recombination instead originates from the sequence between the *loxP* sites. The latter explanation is somewhat plausible, as the T7 terminator is a promoter in the reverse direction and might be enough for the low-level expression of BFP seen before recombination. However, it is still quite a stretch that a weak promoter, facing the wrong direction, would somehow be enough to give any BFP expression. The former explanation is much more plausible and was confirmed through deletion and inversion of the *loxP* site left after recombination (**Figure 9**).

Deleting the *loxP* site restores the sequence to what it was before, which showed expected BFP expression (**Figure 8C**). This provides strong evidence that the *loxP* site is responsible for the reduction in BFP expression. Inversion of the solitary *loxP* site also restores BFP expression, providing further evidence that there is something directional, likely one or both start codons present in the spacer region of the *loxP* site, marked in red: 5' - **ATGTATGC** - 3'. The 3' start codon is in frame with *mTagBFP2*. However, there is a TAG stop codon before the ATG start codon of *mTagBFP2*, making it unlikely that this product is interfering with the expression of BFP. Readthrough of the stop codon also seems unlikely, as previous research has shown that very little readthrough takes place at TAG stop codons, even under conditions that significantly increase readthrough of other stop codons (45).

On the other hand, the 5' start codon is out of frame with *mTagBFP2* and has no stop codon in frame before the start of the BFP gene. The first stop codon is 114 bases downstream, yielding a possible 120 bases ORF, with partial overlap with full-length *mTagBFP2* ORF. The mechanism(s) by which the presence of the *loxP* site silences BFP expression is unclear. Whether it stems from the sequestering of ribosomes (46), RNA interference (47,48), or something else entirely, requires further research.

One thing remains clear: the *loxP* sites have a negative influence on the expression of *mTagBFP2*. The question then becomes, how and why is there a substantial expression of BFP before recombination, as seen in **Figure 8A**? The *loxP* site seems to be enough by itself to stop expression, while there is expression happening if there are two *loxP* sites and two terminators present. The addition of elements designed to negatively affect expression (i.e., terminators) or which have shown to have a negative impact on expression (i.e., *loxP* sites) should never add up to an increase in expression.

Many questions are remaining to be answered and are far beyond the scope of this study. Their relevance to this project is also uncertain, should the BFP switch with both *loxP* sites inverted show expected BFP expression.

### 6.3 Assembly PCR

Purification of each PCR reaction can give a higher yield with fewer unwanted products (49). Each PCR reaction can bring a small amount of template DNA with it, which might be preferentially amplified during the last assembly step. The same primers are used during the last step in the assembly PCR and the original template DNA generation. While it may look from the MACS and sequencing data that the inversion of the *loxP* sites failed with the method used, this is most likely not the case. Instead, what happened is most likely that the reactions all worked and produced the desired PCR products with the correct tail sequences for inversion of the two *loxP* sites. However, one of the reactions (**Figure 11**) was not purified as the ratio of fragment DNA to template DNA was very large. This relative abundance of fragment DNA is offset by the much more effective amplification of the template DNA than the fragment DNA. The difference in amplification efficiency stems from the fact that the template DNA only need two primers to bind, while the fragment DNA need both tails to bind to each other in addition to the two primers. Thus, the desired product with both *loxP* sites inverted is likely present in the reaction but at a minimal concentration compared to the template DNA. As both products are the same size, there is no way of differentiating them before transformation. The end product is a series of PCR reactions that seemingly have worked properly throughout but produce no result. Future work consists of repeating the inversion PCRs for the BFP switch, with thorough removal of any contaminating template DNA. Successful inversion of the *loxP* sites should yield a fully functional BFP switch, which can then be used in conjunction with the pSC101 Cre/YFP plasmid to, finally, be used to study conjugation under different antibiotic conditions.

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## 8. Supplementary information

**Supplementary table 1.** Cycling conditions for Phusion™ High-Fidelity DNA Polymerase.

Temp (°C)	Time	Cycles	<b>Phusion™ High-Fidelity DNA Polymerase</b>	
98	5′	x1	Reagent	Volume
98	30"	x27	dH <sub>2</sub> O	23.7 µl
60	30"		2mM dNTP	5 µl
72	1′30"		5mM F	5 µl
72	7′	x1	5mM R	5 µl
4	∞		5x HF Phusion Buffer	10 µl
			Phusion polymerase	0.3 µl
			DNA template	1 µl

**Supplementary table 2.** Cycling conditions for Phusion™ High-Fidelity DNA Polymerase.

Temp (°C)	Time	Cycles	<b>Phusion™ High-Fidelity DNA Polymerase</b>	
98	5′	x1	Reagent	Volume
98	30"	x2	dH <sub>2</sub> O	22.2 µL
64	30"		2mM dNTP	5 µl
72	2′30"		5mM F	5 µl
98	30"	x2	5mM R	5 µl
62	30"		5x HF Phusion Buffer	10 µl
72	2′30"		DMSO (99%)	1.5 µL
98	30"	x2	Phusion polymerase	0.3 µl
60	30"		DNA template	1 µl
72	2′			
98	30"	x7		
59	30"			
72	2′			
98	30"	x23		
72	2′			
72	7′			
4	∞	x1		

**Supplementary table 3.** Primers used in this study. Red part is primer binding, black is non-binding tails.

Primer	Sequence (5'to 3')	Comment
pEL_galK_Eco_F	TCAGCGACAGCTTGCTGTACGGCA GGCACCAGCTCTTCCG <b>CACACAAC</b> <b>CACACCACAC</b>	Amplification of BFP switch. 40 base pair homology for insertion into <i>galK</i> .
ColiGalKYFPLinR	GTTTGCGCGCAGTCAGCGATATCC ATTTTCGCGAATCCGGAGTGTAAG AA <b>CGCCTTTGAGTGAGCTGATA</b>	Amplification of BFP switch. 40 base pair homology for insertion into <i>galK</i> .
Lox_deletion_F	GCTACTAGAG <b>ACTAGAGAAAGAG</b> <b>GAGAAATACTA</b>	Removal of <i>loxP</i> site.
Lox_deletion_R	TTTCTCTAGT <b>CTCTAGTAGCTAGCA</b> <b>TAATACC</b>	Removal of <i>loxP</i> site.
Lox_inversion_F	CGTATAGCATACATTATACGAAGT TAT <b>ACTAGAGAAAGAGGAGAAAT</b> <b>ACTA</b>	Inversion of <i>loxP</i> site.
Lox_inversion_R	CGTATAATGTATGCTATACGAAGT TAT <b>CTCTAGTAGCTAGCATAATAC</b> <b>C</b>	Inversion of <i>loxP</i> site.
galK_scrn_F	<b>TACGCAAAGTTAACAGTCG</b>	Screening inserts in <i>galK</i> . Amplification of BFP switch.
galK_scrn_R	<b>CAGAGGCGGATAAAAGTG</b>	Screening inserts in <i>galK</i> . Amplification of BFP switch.
ColiGalK_SF	<b>GGTTATGAAATGCTGGCAGA</b>	Screening inserts in <i>galK</i> . Amplification of BFP switch.
ColiGalK_SR	<b>GACTACCATCCCTGCGTTGT</b>	Screening inserts in <i>galK</i> . Amplification of BFP switch.
BFP_Inv_F_Term	CGTATAGCATACATTATACGAAGT TAT <b>TCACACTGGCTCACCTTC</b>	Inversion of <i>loxP</i> site.
BFP_Inv_R1_Term	<b>AAAAAATTAGCGCAAGAAGAC</b>	Inversion of <i>loxP</i> site.
BFP_Inv_R2_Term	CGTATAATGTATGCTATACGAAGT TAT <b>AAAAAATTAGCGCAAGAAGAC</b>	Inversion of <i>loxP</i> site. Same binding part as BFP_Inv_R1_Term, with addition of tail for inversion.
BFP_Inv_R1_AP	<b>GACCGCCGTCGGCCGGAA</b>	Inversion of <i>loxP</i> site. Very high melting temperature.
BFP_Inv_R2_AP	CGTATAATGTATGCTATACGAAGT TAT <b>GACCGCCGTCGGCCGGAA</b>	Inversion of <i>loxP</i> site. Very high melting temperature. Same binding part as BFP_Inv_R1_AP, with addition of tail for inversion.
pEL_pJNEG_F	AATTGTTATCCGCTCACAATTCTCG AGGTGAAGACGAAAGGG <b>CACACA</b> <b>ACCACACCACAC</b>	Insertion <i>aadA</i> and Cre recombinase into pJNEG01. 40 base pair homology for insertion.
pEL_pJNEG_R	GATCTATCAACAGGAGTCCAAGCG AGCTCGTAAACTTGGT <b>CTAACCCT</b> <b>TAGTGACTCCTGC</b>	Insertion <i>aadA</i> and Cre recombinase into pJNEG01. 40 base pair homology for insertion.
OL_galK_scrn_F	<b>ACGGGCGGAAAGTAAAGTC</b>	Screening inserts in <i>galK</i> .
OL_galK_scrn_R	<b>AATGGCGAAGAGAATCAAC</b>	Screening inserts in <i>galK</i> .
VF_2	<b>TGCCACCTGACGTCTAAGAA</b>	Sequencing BFP switch.
BFP_ampl_R	<b>ATGAAGCTGTACATGGAAGG</b>	Sequencing BFP switch.
Ver_YFP_F	<b>CATGCGTAAAGGAGAAGAAC</b>	Screening YFP on pJNEG01
Ver_YFP_R	<b>CATCCATGCCATGTGTAATC</b>	Screening YFP on pJNEG01

**Supplementary table 4.** Digestion using MluI. 37°C for 5 min, heat deactivation 80°C for 5 min

<b>Digestion of pSB4S15 (DA27758)</b>	
<b>ddH<sub>2</sub>O</b>	15 µL
<b>10X FastDigest Green Buffer</b>	2 µL
<b>DNA</b>	2 µL (20 ng)
<b>FastDigest enzyme</b>	1 µL

**Supplementary notes 1**

All sequencing data can be found here:

<https://drive.google.com/drive/folders/1V5W3hzAqRmDSqT2x8WQ31k7uMmyz6sU3>