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# Construction and Evaluation of a Cre-lox-Based Fluorescent Conjugation Tracking System

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# 1. Abstract

Plasmids are small, circular, extrachromosomal double-stranded genetic elements present in bacteria. Plasmids can replicate independently of the bacterial chromosome and play an important role as a transmitter of antibiotic resistance genes between bacteria. Antibiotic resistance genes have been shown to be selected for even in the presence of subinhibitory levels of antibiotics, but the effect of antibiotics on conjugation is not as well understood. To study this, we designed a novel conjugation tracking system utilizing a Cre-expressing plasmid and a chromosomal floxed blue fluorescent protein (BFP) gene. We found that our model worked opposite as intended as cells expressed BFP before conjugation and lost BFP expression upon recombination. An issue with the system was isolated to the direction of the single *loxP* site remaining after recombination. Both *loxP* sites were inverted but this did not restore the intended expression of BFP after recombination. Subsequently the system was modified to increase the space between the promoter region and the single *loxP* site remaining after recombination. This extension produced the desired result as BFP expression now increased upon recombination. Still, further work needs to be done to construct a Cre-expressing plasmid, tune expression of BFP, and show expression of yellow fluorescent protein (YFP) in our model before the system can be applied to clinical isolates.

Keywords: *Antibiotic resistance, horizontal gene transfer, plasmids, conjugation, conjugational frequency, Cre/loxP system*

# 2. Popular Science Summary – "Development of a novel system to track plasmid conjugation."

Bacterial infections have been a major cause of disability and death in human history, but this is thankfully no longer an inescapable fact since the invention of antibiotics. However, we are at risk of backsliding into an era of untreatable infections due to antibiotic resistance, which bacteria develop naturally as a response to our mass use, and misuse, of antibiotics.

One important mechanism by which bacteria become resistant is the spread of plasmids, small circular pieces of DNA which exist independently of the bacteria's own chromosome but inside the bacterial cell. Plasmids can carry one or many genes that provide bacteria with resistance to antibiotics, and can spread rapidly between bacteria causing large populations to become resistant overnight. Infections in patients caused by bacteria carrying multiresistance plasmids can be very difficult, if not nearly impossible, to treat.

When plasmids transmit between bacteria, they do so via a process called conjugation. How plasmids regulate their rate of conjugation in the presence of antibiotics is a topic in need of study. We aim to address this by constructing a novel system to track conjugation of plasmids between bacteria in real time in the presence of antibiotics.

The system works by having bacteria express blue fluorescent protein (BFP), but only in the presence of a plasmid. The plasmid carries a protein called Cre that snips out an inhibitory region of DNA that suppresses BFP expression, thus turning on BFP production from the bacterial chromosome when conjugation occurs. Expression of BFP causes the cell to become fluorescent, which can then be measured by a machine. With this system, we can grow bacteria in the presence of antibiotics at different concentrations to see how the antibiotics affect the frequency of conjugation.

Trials of the system showed that it operated exactly opposite of what was intended; BFP was expressed initially but turned off when conjugation occurred. Previous experiments had indicated that the region of inhibitory DNA that Cre interacts with, called *loxP* sites, could be of issue. An isolated experiment had shown that an inversion of the *loxP* sites would cause the system to function as intended. However, when applied to the actual system, this change did

not resolve the issue. Instead, we found that an extension between a promoter region necessary for BFP to be expressed and one of the *loxP* sites made the system function. Likely due to there being too little space between two different functional sites in the system causing Cre to block transcriptional machinery present at the promoter.
A breakthrough has occurred in the development of our system, expression of BFP is now functioning as intended: Bacteria are BFP-negative before conjugation and turn BFP-positive when the plasmid is introduced. Nevertheless, work still remains to calibrate the BFP expression to ensure that robust data will be produced when the system is tested in a live environment.
We remain hopeful that before long we will see the system employed in pathogenic bacteria isolated from real clinical cases and the resistance plasmids they interact with.

# 3. Introduction

## 3.1. Antibiotic Resistance

Antibiotics have since their discovery in the 1920s and further development taken on the role of Atlas, a proverbial giant holding up our world of high standard medical care and exemplary quality of life. It is difficult to imagine what the 21st century would be like without the advances in healthcare and increase of life expectancy brought on by the use of antibiotics in the last ~80 years. We are however on our way into a post-antibiotic era brought on by our indiscriminate usage of antibiotics, lack of development of new classes of antibiotics, and antibiotic resistance development, both in pathogens and non-pathogens (1).
According to a worst-case calculation, antibiotic resistance will cause an excess of 10 million deaths yearly by 2050, which is a higher global mortality than caused by cancer today. Antibiotic resistance is predicted to negatively affect five out of the 17 UN sustainable development goals, including general health, food security, responsible consumption & production, clean water & sanitation, and poverty & inequality (2). Bacteria which are exposed to antibiotics habitually develop resistance to the encountered antibiotics, compromising future treatments with similar agents. Antibiotic resistance was observed and its spread postulated already by the discoverer of penicillin himself, Alexander Fleming (3). Fleming noted in his 1945 Nobel lecture that:

> *"[But I would like to sound one note of warning. Penicillin is to all intents and purposes non-poisonous so there is no need to worry about giving an overdose and poisoning the patient. There may be a danger, though, in underdosage.]* ***It is not difficult to make microbes resistant to penicillin in the laboratory by exposing them to concentrations not sufficient to kill them, and the same thing has occasionally happened in the body****."*

Today we are knowledgeable about the cellular mechanisms of antibiotic resistance which can be divided into six main categories (4): (I) Modifying the cellular target of the antibiotic, reducing or abolishing the drug's affinity for the target, (II) decreased drug uptake, e.g. downregulation of porines or modification of cell wall structure, (III) activation of efflux machinery to reduce the concentration of antibiotic in the cell, (IV) global changes in metabolic pathways to circumvent the antibiotic's effect, (V) secretion of compounds that modify or degrade antibiotic compounds, and (VI) overproduction of the cellular target generating a surplus that retains normal function. Resistance genes that fulfill these different functions can be present both on the bacterial chromosome and on extrachromosomal elements such as plasmids (5). This difference in location affects the stability of the resistance genes in a

population and the likelihood of spreading by horizontal transfer. For example, genes that fulfill an essential need in the bacterium are much more likely to be found on the chromosome than on a plasmid. Therefore mutations in these genes are also more commonly found in the chromosome where they can e.g. lessen the affinity of drugs to their target(s) in the bacterium.

### 3.2. Minimal Inhibitory Concentration

Like most drugs, antimicrobials are dosed to specific concentrations in order to obtain an effect in the patient with the least possible amount of side effects. One of the most important concentrations for clinical use is the minimum inhibitory concentration (MIC). MIC is defined as the lowest concentration needed to inhibit all visible growth of a bacterium and is determined using *in vitro* conditions (6). For a long time it has been assumed that selection for resistant bacteria principally begins when the antibiotic concentration rises above the MIC of the susceptible population, at which point non-resistant bacteria are unable to grow (7–9). However, a body of research now shows that selection for resistance occurs even at sub-MIC concentrations in the interval between minimum selective concentration (MSC) and MIC (**Figure 1**) (10–13). MSC is defined as the concentration at which a resistant strain can outcompete its non-resistant counterpart. The concentration value at which this occurs is dependent both on the relative fitness of the strains present in an environment, as well as environmental factors themselves (14).

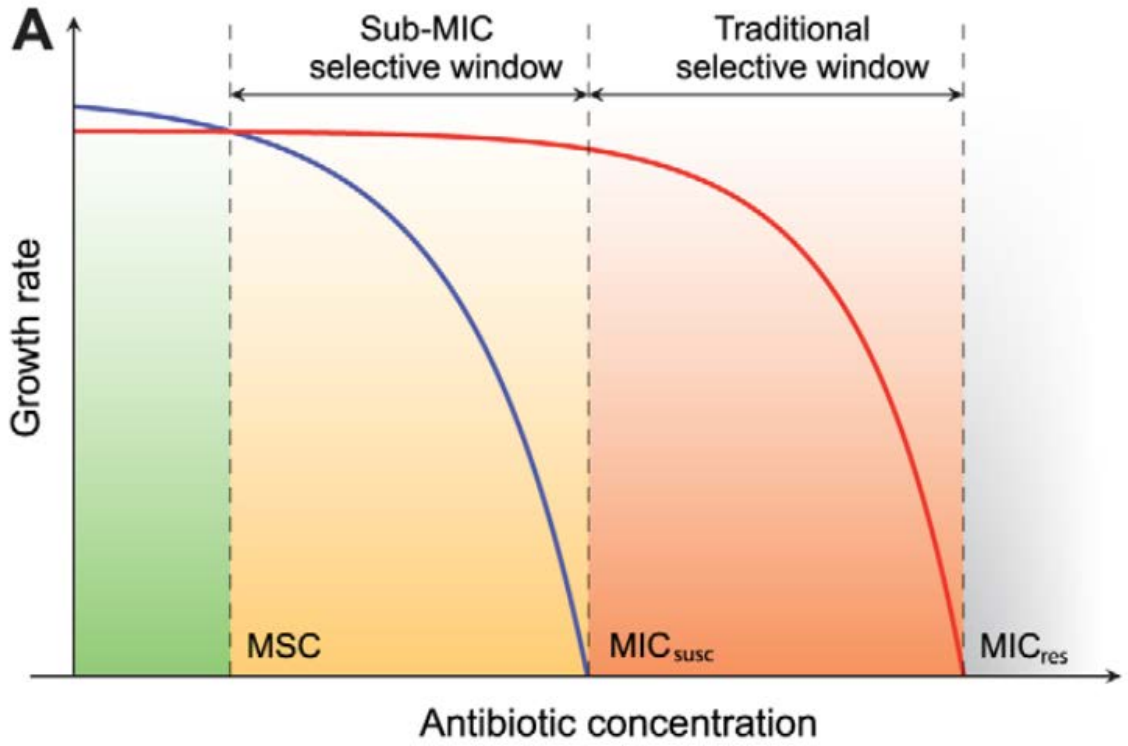


**Figure 1. Bacterial growth rate as a function of antibiotic concentration.** The blue and red lines indicate the relative growth rate of a susceptible and resistant strain, respectively. The green background indicates a low level of antibiotics where the susceptible strain outcompetes the resistant one. This relationship is inverted in the yellow area and in the red only the resistant strain is able to grow. MSC = Minimum Selective Concentration; $MIC_{susc}$ and $MIC_{res}$ = Minimum Inhibitory Concentration susceptible/resistant strain. Adapted from Gullberg et al. (10).

An essential concept when discussing MIC and MSC is the growth disadvantage or “fitness cost” that resistant strains incur due to the mutations and/or additional genes that provide resistance. Even if not always the case, resistant strains often experience a dual effect of both being resistant to antimicrobial(s) as well as having a reduced growth rate due to the mechanism

that provides resistance. Resistant populations are put at a relative disadvantage in absence of the selective antimicrobial and this might cause the resistant strain to revert to a non-resistant state or be outcompeted by a more fit, non-resistant strain due to the difference in growth rate. Important to note however, is that many bacteria exhibit secondary, compensatory mutations which reduce the fitness cost associated with a previous mutation, including resistance mutations (15). This process of secondary mutation to alleviate fitness costs is sometimes so successful that the fitness cost caused by the original mutation becomes negligible or disappears completely (16).

### 3.3. Horizontal Gene Transfer and Plasmids

Bacteria are known to share genetic material and genes directly between individual cells by a mechanism called Horizontal Gene Transfer (HGT). HGT is distinguished from ordinary vertical transfer which invariably includes cell reproduction and heritage. Usually the sharing of genes via HGT is more frequent between members of the same species, but HGT across larger phylogenetic distances and species barriers has been known to occur (17, 18). This includes transferrable genetic elements that are not necessarily defined as part of the core genome of any particular species and when introduced to an individual can alter the phenotype of the cell significantly. There are three main ways which genes can transfer horizontally between individual bacterial cells both in the natural environment and in a lab setting: Transformation, transduction, and conjugation (19, 20).

Transformation is the process of uptake, stabilization, and recombination of free DNA from the environment into the host chromosome. This necessitates a bacterial cell that is naturally competent, which is not a ubiquitous trait in bacteria (19). Environmental conditions, such as stress put on bacteria by the presence of antibiotics, can also affect the rate of successful transformation (21).

Transduction involves bacterial viruses, bacteriophages, that “mistakenly” package bacterial DNA during their life cycles. The bacteriophage later injects the packaged DNA into a different bacterium wherein the DNA can be recombined into the host genome, given that the sequences are sufficiently similar.

Lastly, conjugation involves conjugative plasmids, extrachromosomal genetic elements that replicate independently of the bacterial chromosome and encode gene products that form physical bridges between bacterial cells. Once a connection is established, the plasmid inside the donor cell copies itself and sends the copy over to the recipient cell. Thus increasing the count of the plasmid and cells inhabited by the plasmid both by one. Plasmids do not only encode genes for their propagation but also products that can radically alter the phenotype of the cell. For example, genes that in bacteria modulate cell metabolism (22), add virulence factors (23), or provide antibiotic resistance (11). The MultiDrug Resistant (MDR) plasmids are a type of plasmid which can encode and express resistance genes for multiple antibiotics, biocides, and heavy metals simultaneously (11).

An important feature of MDR-plasmids is that the presence of *any* of the toxic compounds it protects against will usually select for the entire plasmid and therefore all resistances at once. This has been observed even at very low concentrations of antibiotics, well below the MIC for a clone of *E. coli* lacking the MDR-plasmid (11). Importantly, whether by plasmid conjugation or other means, HGT provides a powerful tool for advantageous genes, such as antibiotic resistance genes, to spread within and between bacterial populations at a rapid rate.

Antibiotics act on bacteria as an environmental stressor (24). There is an ongoing debate of how important antibiotics are as a signal molecule to promote HGT and which mechanisms that are important for the transmission of plasmids (25–27). Many experiments quantify transconjugants at different timepoints in the presence of antibiotics, then draw conclusions about the effect antibiotics have on conjugational efficiency. This methodology of counting

transconjugants has the drawback that it cannot tease apart the two factors that feed into conjugational efficiency: One being the rate of conjugational events, and the other the differing growth rates of donors, recipients, and transconjugants during the experiment (25, 28). Furthermore, a study by Lopatkin et al. showed that during laboratory conditions, even with antibiotics present, the physiological state of bacterial cells or access to nutrients influenced conjugational frequency to a much larger extent than the presence of an antibiotic (25). This must be accounted for both when designing experiments meant to measure conjugational efficiency and when comparing results from different studies.

### 3.4. pUUH239.2

Between 2005 and 2010, an extended nosocomial outbreak of a single multiresistant ESBL-producing clone of *Klebsiella pneumoniae* occurred at Uppsala University Hospital, Sweden (17). During the outbreak, 320 patients were infected or colonized with the clone (Birgitta Lytsy, UUH, personal communication). The spread was largely attributed to patient-to-patient contact and most of those affected were elderly and/or immunocompromised. A large (220 kbp) multiresistance IncFII plasmid (later named pUUH239.2) was present in the outbreak clone and encoded resistance against β-lactams ($bla_{\text{TEM-1}}$, $bla_{\text{OXA-1}}$), macrolides [*mphR*(A), *mrx*, and *mph*(A)], tetracyclines [*tet*(A), *tetR*], aminoglycosides [*aac-(6')-1b-cr*, *aadA2*], trimethoprim (*dhfrXII*), sulphonamides (*sul1*), quaternary ammonium compounds (*qacED1*), as well as heavy metal ions including silver, copper, and arsenic. Remarkably, the study by Sandegren et al. which sequenced and characterized pUUH239.2 found that the plasmid had horizontally transferred from *K. pneumoniae* to *E. coli* in the intestinal flora of patients on multiple separate occasions. These findings underscore the importance of elucidating and understanding plasmid dynamics when bacteria are exposed to antibiotics.

### 3.5. Cre/loxP Recombination

The Cre/loxP recombination system consists of a Cre recombinase protein (***C**reates **Re**combination* or ***C**yclization **Re**combination*) and one or multiple *loxP* (**l**ocus **o**f **cross**over, **p**hage) recombination sites that when combined can cause excisions, inversions, integrations, and translocations between predetermined sites in DNA (29). Cre/loxP was initially discovered in the P1 bacteriophage where Cre/loxP circularizes the P1 genome post-infection (30) and also unlinks interlocked P1 plasmids (31) in host cells as a part of the phage life cycle. The Cre/loxP system is very efficient both in eukaryotic and prokaryotic cells and has no need for additional host proteins or co-factors to perform recombination. Recombination occurs in both linear and supercoiled DNA substrates. The Cre/loxP system has been extensively adapted to work in mice (29) as well as in bacterial (32) and plant (33) models. The Cre protein is 38 kDa in size and a member of the Int family of tyrosine site-specific DNA recombinases. It catalyzes a conservative, meaning that no nucleotides are lost or added, site-specific recombination between *loxP* sites in the genome (29).

*LoxP* sites are 34 bp in size (5' - ATAACTTCGTATAATGTATGCTATACGAAGTTAT - 3'), consisting of two 13 bp inverted repeat elements surrounding an 8 bp asymmetrical core/spacer region (29). The asymmetry in the core/spacer region is important since it bestows every *loxP* site with a direction. Cre binds to the inverted repeat elements and the core/spacer region is where the double stranded breakage and rejoining of DNA occurs. Depending on the configuration and location of *loxP* sites present, the Cre recombinase can catalyze a range of different reactions: (I) Two unidirectional *loxP* sites on the same DNA fragment will cause an excision of the flanked DNA segment as a closed circular molecule that is usually not maintained, leaving behind only a hybrid of the two *loxP* sites. (II) Two *loxP* sites with opposite directions will cause an inversion of the flanked DNA. (III) Two unidirectional *loxP* sites on different DNA fragments, e.g. a chromosome and plasmid, will cause a reciprocal translocation

to occur if both fragments are linear and an integration if at least one fragment is circular. If a segment of DNA, e.g. a gene, is flanked on both sides by *loxP* sites it is said to be floxed. Cre/loxP recombination provides a good basis for a model to track conjugation events accurately. When combining a floxed reporter gene in a recipient strain and a conjugative Cre-expressing plasmid from a donor, a successful conjugation will introduce Cre to the cell and turn the reporter gene on. This allows for each conjugation event to be tracked by measuring the output of the reporter gene.

**Aim**

To construct a novel bacterial conjugation tracking system utilizing the Cre/loxP system and to use the tracking system to evaluate the effects of sub-MIC concentrations of antibiotics on conjugation frequency between bacteria. This system, when successfully developed and applied, will help us understand how antibiotic resistance spreads via plasmids and become a tool to counteract the global problem of antimicrobial resistance in human pathogens.

# 4. Materials and Methods

## 4.1. Bacterial strains

Most strains used in this study were derived from *Escherichia coli* MG1655. 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 40 mg/L; kanamycin 50 mg/L; spectinomycin 50 mg/L; tetracycline 15 mg/L; trimethoprim 75 mg/L; and zeocin 50 mg/L.

Sigma-Aldrich supplied all antibiotics with the exception of zeocin which was supplied by Invitrogen, MA, USA.

***Table 1**. Bacterial strains*

| Strain | Genotype | Comment |
|---|---|---|
| DA4201 | Eco MG1655 | Wildtype MG1655 strain. |
| DA21802 | Eco NEB 5-alpha / pSB1A2-J61047 (AmpR, Cre). Clone 1. | Cre recombinase plasmid, ampicillin resistant, lower level of Cre-expression than DA27758 due to no dedicated promoter driving Cre. |
| DA24100 | Eco MG1655 /pSIM5-Tet lambda red system TetR | Lambda red plasmid, tetracycline resistant. |
| DA25015 | Eco MG1655 argH-ssrA tag /pSIM5 /pJNEG01 (miniF, YFP-bla(del bp 98747-44123)) | Lambda red plasmid. YFP on conjugative plasmid pJNEG01. |
| DA25827 | DH5a / pSB3S5-SYFP2_opt-B1006 | Biobrick compatible plasmid with YFP and terminator in the cloning site. Spectinomycin resistant. |
| DA26550 | Eco DH5alpha / pSB8K15-J23101+B0034+SYFP2_opt+B1006 | YFP on plasmid, kanamycin resistant. |

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| DA27758 | Eco NEB 5-alpha / pSB4S15-J23116-B0032-Cre | Cre recombinase plasmid, spectinomycin resistant. |
| DA28200 | Eco galK::FRTscar-J23101-SYFP2 | Constitutively active chromosomal YFP. |
| DA28202 | Eco galK::FRTscar-J23101-mTagBFP2 | Constitutively active chromosomal BFP. |
| DA28851 | Sty LT2 galK::kan-new_lox_switch_mTagBFP2 /pSIM5-tet | BFP lox-switch, kanamycin resistant. |
| DA32004 | Eco galK::kan-new_lox_switch_mTagBFP2/pSIM5-tet | BFP switch from DA28551 inserted in *galK*. |
| DA32305 | Eco K12 MG1655 F- [Lambda cI857, (attB-orf60a)::tetAR(sw), DEL(cro-bioA)], dapA::Kan Clone 1 | Requires 2,6-Diaminopimelic acid to grow. |
| DA46327 | Eco MG1655 / pDA_VaC::yfp | YFP plasmid, chloramphenicol resistant. |
| DA75355 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites. Clone 1. |
| DA75357 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites. Clone 2. |
| DA75359 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites. Clone 3. |
| DA75405 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators not present in construct. |
| DA75409 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators present in construct. |
| DA76330 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators not present in construct. J23-101 promoter exchanged for J23-105. |
| DA76331 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators not present in construct. J23-101 promoter exchanged for J23-106. |
| DA76332 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators not present in construct. J23-101 promoter |

| | | |
|---|---|---|
| | | exchanged for J23-114. Clone A. |
| DA76333 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators not present in construct. J23-101 promoter exchanged for J23-114. Clone B. |
| DA76334 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators not present in construct. J23-101 promoter exchanged for J23-118. |
| DA76335 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators present in construct. J23-101 promoter exchanged for J23-105. |
| DA76336 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators present in construct. J23-101 promoter exchanged for J23-110. |
| DA76337 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators present in construct. J23-101 promoter exchanged for J23-114. Clone A. |
| DA76338 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators present in construct. J23-101 promoter exchanged for J23-114. Clone B. |
| DA76339 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, terminators present in construct. J23-101 promoter exchanged for J23-115. |
| DA76340 | Eco MG1655 | BFP lox-switch with inverted *loxP* sites and a prolonged promoter region, |

| | | |
|---|---|---|
| | | terminators present in construct. J23-101 promoter exchanged for J23-118. |
| No DA # | Eco MG1655 / pSC101_YFP110_Cre | Cre recombinase and YFP plasmid, kanamycin and spectinomycin resistant. |

### 4.2. Polymerase chain reaction

All PCR reactions were performed using Phusion™ High-Fidelity DNA Polymerase (Thermo Fisher Scientific Inc., MA, USA) on a C1000 Touch™ Thermal Cycler (Bio-Rad, CA, USA) and an S1000 Thermal Cycler (Bio-Rad). PCR 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 visualized under UV light, and photographed using a Gel Doc XR+ (Bio-Rad) with accompanying software (Quantity One 4.6.5., Bio-Rad). GeneRuler 1 kb and 50 bp DNA ladders (Thermo) were used to determine band sizes. GeneJET Gel Extraction Kit (Thermo) was used to purify PCR products according to the manufacturer's instructions either from the electrophoresis gel or from the PCR product directly. Gel and PCR products were eluted in provided elution buffer or sterilized, deionized water and stored at -20ºC.

### 4.3. The BFP lox-switch

The DA32004 strain was previously constructed by amplifying the mTagBFP2 cassette from DA28851 using the primers pEL_galK_Eco_F and ColiGalKYFPLinR and inserting the resulting product into DA24100 using lambda red recombineering (LRR) (**Figure 2**). The functional aspects of the BFP lox-switch present in DA32004 consist of the J23-101 promoter (BBa_J23101, Registry of Standard Biological Parts) and its associated RBS (BBa_B0034) capable of driving expression of *mTagBFP2*. In the construct, the two terminators T7 (BBa_B0012) and lambda T1 terminator (BBa_K864601) point in the forward direction and stop any latent expression. The terminators are flanked by *loxP* sites which allows them to be removed from the construct by Cre recombinase, allowing for expression of *mTagBFP2*.

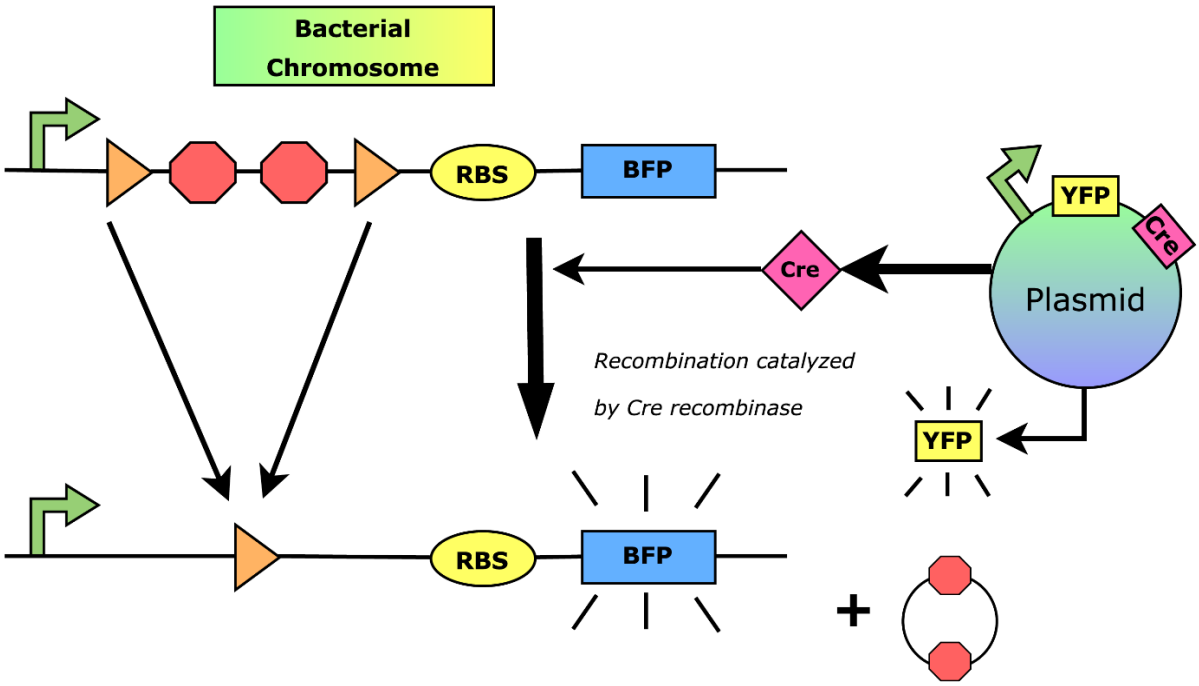

**Figure 2. BFP lox-switch in DA32004.** Two terminators (red) stopping expression of BFP are flanked by *loxP* sites (orange) in the chromosome. Upon recombination by Cre, the terminators are removed and BFP is expressed.

### 4.4. Inversion of the loxP sites

In order to avoid unwanted primer binding from DA32004 that is not part of our construct, the BFP lox-switch was amplified using the primers ColiGalK_SR and galK_scrn_R. The cycling conditions are presented in **Supplementary Table 1.** The resulting product was used directly and indirectly in ten additional PCR reactions using previously attained PCR products and a plurality of primers (**Table 2**). Using the amplified BFP lox-switch from DA32004, four 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. 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. 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 before storing at -20ºC. Product from reaction (10) was used for lambda red insertion into DA24100 giving rise to DA75355, DA75357, and DA75359.

*Table 2: The DNA templates, primers, and cycling conditions used to invert the loxP sites.*

| Reaction # | DNA template | Primer (F) | Primer (R) | PCR cycling conditions |
|---|---|---|---|---|
| Reaction 1 | DA32004 amplified BFP lox-switch | ColiGalK_SR | Lox_inversion_R | Supplementary table 1 |
| Reaction 2 | DA32004 amplified BFP lox-switch | BFP_Inv_F_Term | BFP_Inv_R1_Term | Supplementary table 1 |
| Reaction 3 | DA32004 amplified BFP lox-switch | Lox_inversion_F | GalK_scrn_R | Supplementary table 1 |
| Reaction 4 | DA32004 amplified BFP lox-switch | BFP_Inv_F_Term | BFP_Inv_R1_AP | Supplementary table 1 |
| Reaction 5 | Products from reaction 1 & 2 | ColiGalK_SR | BFP_Inv_R1_Term | Supplementary table 2 |

| | | | | |
|---|---|---|---|---|
| Reaction 6 | Products from reaction 1 & 4 | ColiGalK_SR | BFP_Inv_R1_AP | Supplementary table 2 |
| Reaction 7 | Product from reaction 5 | ColiGalK_SR | BFP_Inv_R2_term | Supplementary table 1 |
| Reaction 8 | Product from reaction 6 | ColiGalK_SR | BFP_Inv_R2_AP | Supplementary table 1 |
| Reaction 9 | Products from reaction 3 & 7 | galK_scrn_F | ColiGalK_SF | Supplementary table 2 |
| Reaction 10 | Products from reaction 3 & 8 | galK_scrn_F | ColiGalK_SF | Supplementary table 2 |

### 4.5. Elongation downstream of J23-101 promoter

The BFP lox-switch was amplified from the previously produced DA75355 and DA75357 strains containing the inverted *loxP* sites using the ColiGalK_SR and galK_scrn_R primers with the cycling conditions from **Supplementary Table 1**. The elongated promoter region BFP lox-switch was produced in two separate fragments using the primer pairs ColiGalK_SR & Distpromo_loxP R, and galk_scrn_R & Distpromo_loxP F, respectively, with the cycling conditions from **Supplementary Table 2**. The complete product was produced by combining 0.5 µL purified DNA from both fragments in a PCR reaction using the primers galk_scrn_F and ColigalK_SF and the cycling conditions from **Supplementary Table 2**. All three previously mentioned reactions were analysed using gel electrophoresis and the band matching the intended size was isolated and purified using a GeneJET Gel Extraction Kit (Thermo) to be used as template for a following reaction. The combined fragment was later inserted into DA24100 using lambda red recombineering producing the strains DA75405, DA75406, DA75407, DA75408, DA75409, and 75410. DA75355 gave rise to DA75405, DA75406, and DA75407. DA75357 was used to produce DA75408, DA75409, and DA75410. The elongation of 26 basepairs in the BFP lox-switch was verified by sequencing (Eurofins Genomics, Ebersberg, Germany).

### 4.6. Exchanging the J23-promoter

DA75405 and DA75409 containing the inverted *loxP* sites and prolonged promoter region were amplified using the ColiGalK_SR and galK_scrn_R primers with the cycling conditions from **Supplementary Table 1**. A collection of PCR primers with complementary overhangs were designed to overwrite the original J23-101 promoter with sequences matching the following promoters from the J23 family: 105, 106, 110, 114, 115, 118 (**Figure 3**). The first half of the construct was produced in six different replicates using the primers ColiGalK_SR and prol_J23-105_R, prol_J23-106_R, prol_J23-110_R, prol_J23-114_R, prol_J23-115_R, and prol_J23-118_R, respectively. The second half of the construct was produced also in six replicates using the primers GalK_scrn_R and prol_J23-105_F, prol_J23-106_F, prol_J23-110_F, prol_J23-114_F, prol_J23-115_F, and prol_J23-118_F, respectively. The corresponding halves of the construct were combined into one, separately, using the primers galK_scrn_F and ColigalK_SF. All PCRs other than the amplification of the entire BFP lox-switch were performed using primers at 10 µM concentration and the PCR cycling conditions found in **Supplementary Table 2**. Resulting strains were analysed in a magnetic-activated cell sorting instrument (MACS) capable of reading bacterial fluorescence and used for plasmid transformation reactions. The replacement of the promoter was verified by sequencing in all produced strains (Eurofins).

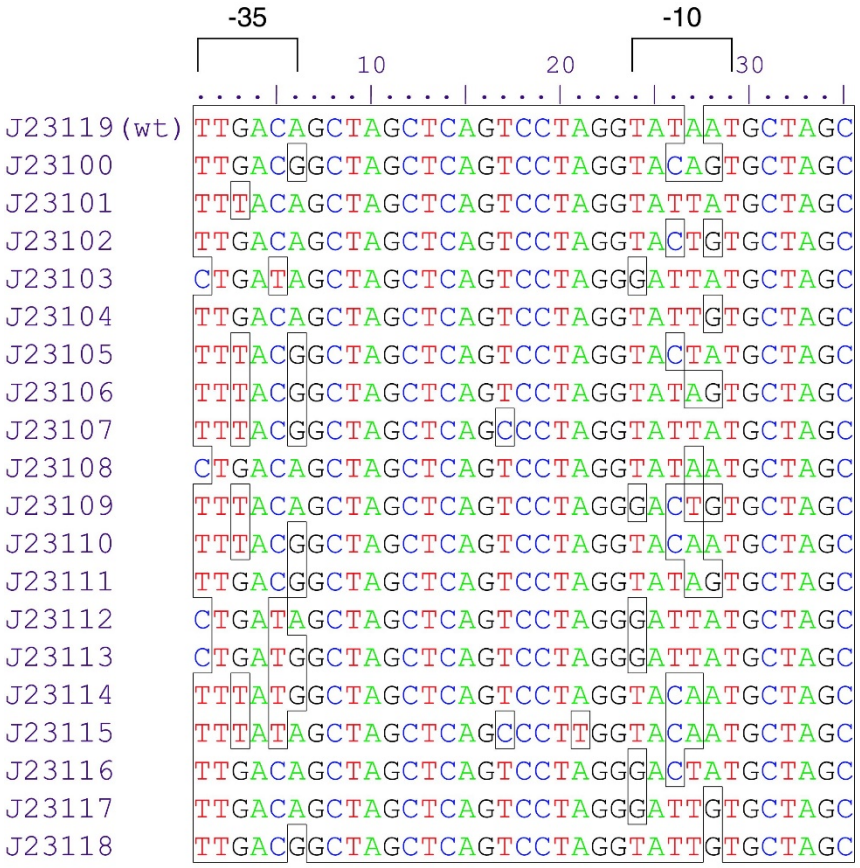


**Figure 3. The J23 promoter family.** Indicated are the bases which differentiate the members of the family. J23-119 is the "consensus" promoter sequence and also the strongest in the family. Image from parts.igem.org.

### 4.7. Lambda red recombineering

Lambda red recombineering was performed as previously described by Datsenko and Wanner (34). DA24100 was grown in liquid LB culture overnight at 30ºC 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 30ºC and 200 r.p.m. shaking until $OD_{600} \approx 0.2$-0.3. Red expression was induced by placing cultures in a 42ºC 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 continuously kept on ice and washed three times in cold 15 mL 10% glycerol (VWR International, PA, USA) using a Mega Star 1.6R centrifuge (VWR) set to 4ºC and centrifuged at 4200 x *g* for 10 minutes for each wash. Finally, to prepare them for electroporation, the cells were centrifuged a final time using the same r.p.m. and duration and resuspended into 0.5-1.0 mL of 10% glycerol. 40 µL of cells and up to 200 ng purified PCR product were mixed and transferred to 1 mm gap electroporation cuvettes (VWR). Electroporation was performed 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 $MgSO_4$, and 20 mM glucose) pre-warmed to 37ºC and were allowed to recover for at least 3h or overnight at 30ºC and 200 r.p.m. shaking before plating on selective media containing 50 mg/L kanamycin at 30ºC. Prepared DNA was stored at -20ºC and thawed on ice in preparation for electroporation.

### 4.8. Plasmid DNA preparation

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 concentrations were determined using a NanoDrop 1000 spectrophotometer (Saveen Werner, Malmö, Sweden), and the purified plasmids were stored at -20ºC.

### 4.9. Plasmid transformation

Cells were grown overnight in liquid LB at 30ºC with 200 r.p.m. shaking. 500 µL of overnight culture was added to 50 mL LB and put at 37ºC 200 r.p.m. until $OD_{600}$ reached 0.5-0.6. Cells were put on ice for 10 minutes and afterwards poured into pre-chilled 50 mL falcon tubes and centrifuged at 4500 r.p.m. for 10 minutes at 4ºC. Cells were washed in 40 and 25 mL 10% autoclaved glycerol and centrifuged for 10 and 15 minutes respectively at 4500 r.p.m.. A final wash in 2 mL glycerol and centrifugation for 20 minutes at 10,000 r.p.m. was performed before cells were resuspended in 1 mL glycerol and aliquoted 50 µL in clean Eppendorf tubes before electroporation with the addition of 1 µL purified plasmid and the same settings as for lambda red recombineering described previously. After electroporation, cells were transferred to 1 mL of SOC medium that had been pre-heated to 37ºC and incubated for 3h or overnight at 30ºC 200 r.p.m. before plating with appropriate selective antibiotics.

### 4.10. Conjugation of bacterial cells

Recipient and donor cells were grown overnight in liquid LB at 37ºC, 200 r.p.m. shaking with addition of 2,6-Diaminopimelic acid (2,6-DAP) (Sigma-Aldrich, MO, USA) and antibiotics as needed to retain the 2,6-DAP dependent strains and conjugative plasmids. 2,6-DAP was dissolved in sterilized, deionized water to a stock concentration of 10 mg/L and added to a final concentration of 20 mg/L. 500 µL of overnight culture was added to 50 mL of LB containing DAP and antibiotics when relevant and incubated at 37ºC for 2h. 100 µL of the donor strain was mixed with 1000 µL of the recipient strain. 50 µL of the mix was spotted onto LB plates containing DAP and relevant antibiotics. Plates were incubated right side up at 37ºC overnight. The spots were resuspended in 1 mL Phosphate Buffered Saline (PBS). 100 µL was plated directly as well as $10^{-1}$ and $10^{-2}$ serial dilutions. All plates were incubated at 37ºC overnight. Resulting colonies were restreaked, analysed in MACS, and when relevant used for further conjugations.

### 4.11. Fluorescence analysis

Cells were grown overnight at 30ºC and 200 r.p.m. shaking with appropriate antibiotics. 1 µL of overnight culture was diluted in 200 µL 0.2 µm filtered PBS in 96-well plates or 5 µL of cell culture in 1 mL of 0.2 µm filtered PBS in single-use tubes. Cells were allowed to develop fluorescence for a minimum of 20 minutes at room temperature before analysis using a MACSQuant®VYB (Miltenyi Biotec, Bergisch Gladbach, Germany).

### 4.12. 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 a GeneJET Gel Extraction Kit (Thermo) following instructions aimed at producing a product to be sequenced. DNA concentration was measured using the 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 lox-switch was sequenced using four primers: BFP_ampl_R, ColiGalK_SR, VF_2, and galK_scrn_R. Qiagen CLC Main Workbench 21.0.5 (QIAGEN, Aarhus A/S, Denmark) was used to do alignments and analyse sequencing data.

# 5. Results

## 5.1. BFP lox-switch constructs with inverted loxP sites show BFP expression opposite of design

Previous work in the lab had been performed in order to study the Cre-lox system, and some unexpected results were found. DA32004 has two floxed inhibitors that would inhibit expression of blue fluorescent protein (BFP) until a recombination event catalyzed by Cre occurred. The recombination event would remove the inhibitors and allow for expression of BFP. DA32004 had its fluorescence measured using MACS both before and after transformation with a Cre-expressing plasmid. MACS showed that opposite to what was expected, DA32004 clearly expressed BFP before recombination and lost BFP expression upon recombination (data not shown). It was suspected that the single *loxP* site remaining after recombination, more specifically two ATG-codons present within the *loxP* site, could be interfering with BFP expression. To investigate this, DA32004 was transformed with the Cre-expressing plasmid pSB1A2 (DA21802). Using assembly PCR, the remaining *loxP* site was then either inverted or removed. Both inversion and removal of the *loxP* site remaining after recombination produced a strong BFP signal as measured by MACS (data not shown). It was concluded that the source of error had to be a direction-dependent element present inside the *loxP* site. The interfering element had to be both (A) present in the *loxP* site and (B) direction-dependent; or else inversion and deletion would not both have restored BFP expression in the strain.

The current project aims to resolve the problems and construct a strain with the BFP lox-switch functioning correctly. An experiment was designed to invert both *loxP* sites in the BFP lox-switch, but keep the direction of the terminators. This would create a construct that when recombined, the remaining *loxP* site would have an inverted direction and allow for expression of BFP. An assembly PCR protocol was carried out in four steps and included ten total PCRs. All PCRs were performed with accompanying gel band purification to remove lingering template DNA which might otherwise interfere with the assembly PCR. The resulting final product was then inserted into DA24100 using lambda red recombineering, producing the strains DA75355, DA75357, and DA75359.

BFP expression was analysed using MACS (**Figure 4**). Unexpectedly, MACS showed a very strong BFP expression, already before recombination, in two of the recombineered strains; DA75355 and DA75359 (**Fig. 4A)** (data not shown for DA75359). More in line with expectations, only a weak BFP expression was shown in the third strain; DA75357 (**Fig. 4B**). DA28202 with its constitutively active *mTagBFP2* was measured as a reference and was shown to be strongly BFP-positive (**Fig. 4C**). After transformation with the Cre-expressing plasmid pSB4S15 (DA27758), MACS showed that both DA75355 and DA75357 completely lost their BFP expression (**Fig. 4D, 4E**).

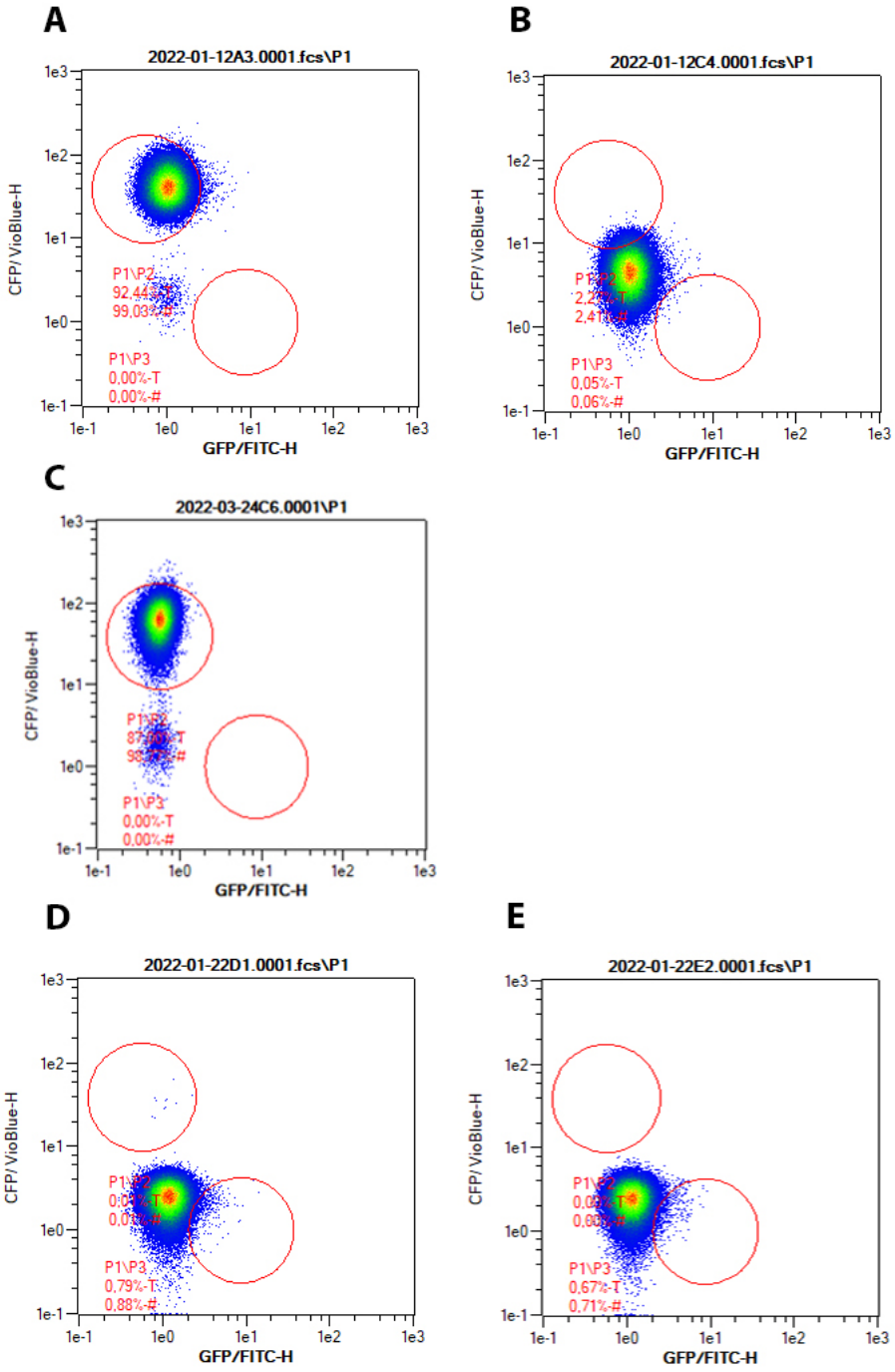


**Figure 4. DA75355 and DA75357 containing the BFP lox-switch with inverted *loxP* sites show a loss of BFP expression when transformed with the Cre-expressing pSB4S15 plasmid.** 1 µL of overnight cell culture was diluted in 200 µL of filtered PBS and incubated at room temperature for a minimum of 20 minutes before analysis using MACS. A minimum of 100,000 cells were counted. The Y-axis shows BFP expression. (A) DA75355 is a strong expressor of BFP before addition of Cre. (B) DA75357 is a weak expressor of BFP before addition of Cre. (C) DA28202, a strain with constitutively active BFP, showing the expected level of BFP expression after recombination. (D, E) Transformation with the Cre-expressor pSB4S15 (DA27758), reduces expression of BFP in both DA75355 and DA75357 to a very similar level.

The DA75355, DA75357, and DA75359 strains were all produced using the same final product from the assembly PCR that inverted the *loxP* sites. However, different replicates were set up

by using different volumes of PCR product added to an equal amount of DA24100 cell culture during lambda red recombineering.
Sequencing of DA75355 showed a deletion between the *loxP* sites in the construct, indicating that a recombination event had occurred without addition of Cre (**Figure 5**). DA75357 was shown to carry the whole BFP lox-switch with the inverted *loxP* sequences as intended. Additional sequencing after transformation of DA75355 and DA75357 with the pSB4S15 Cre-expressing plasmid showed that in DA75357 the recombination event had occurred as designed. The terminators had been removed and left was a single inverted *loxP* site. No change was seen in DA75355, as recombination had already taken place.

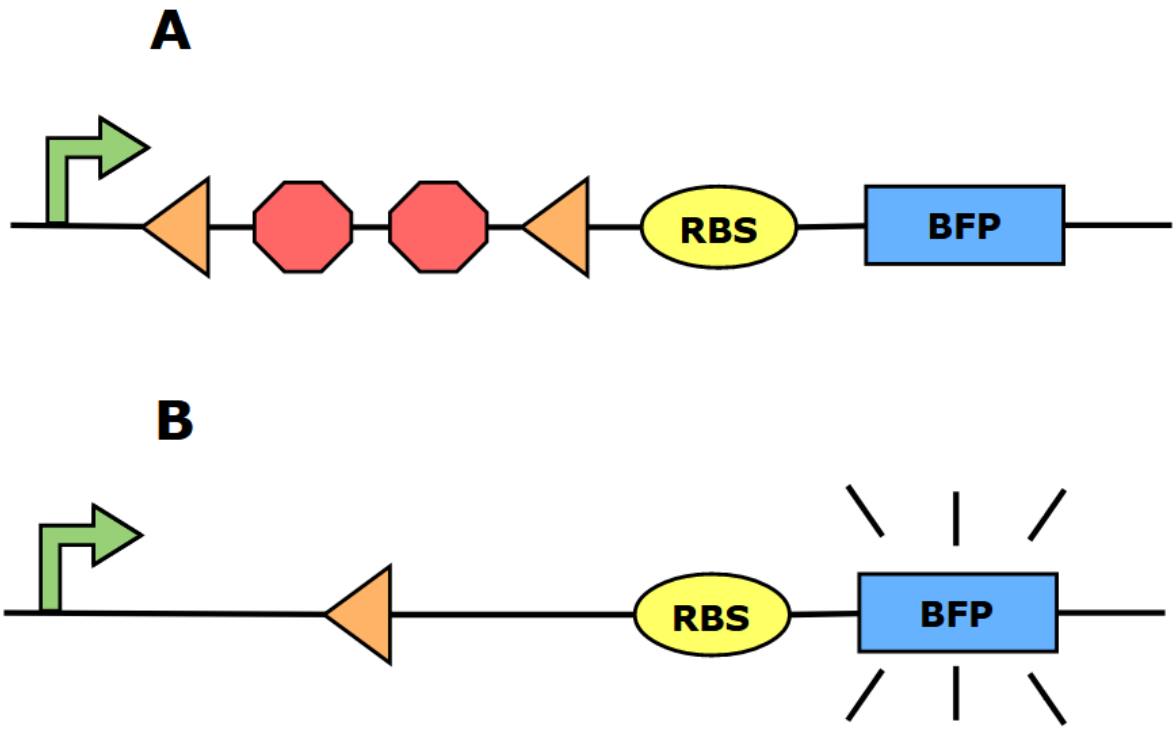


**Figure 5. The BFP lox-switch as present in (A) DA75357 and (B) DA75355 before addition of Cre.** Sequencing showed that (A) the *loxP* sites had been successfully inverted in DA75357 and that (B) recombination had occurred prematurely without addition of Cre in DA75355. In the latter case removing the terminators and inducing BFP expression.

There seemed to be an effect where a recombination event could occur prematurely, possibly during the lambda red recombineering insertion into DA24100. In the case of DA75355, this caused removal of the terminators and a strong expression of BFP prior to Cre transformation. Unexpectedly, the MACS results for the correctly constructed strain, DA75357, showed that the inversion of the *loxP* sites did not solve the problem with the expression of BFP before addition of Cre.
Furthermore, the BFP expression after recombination with Cre did not increase, as expected, but was instead reduced even with inverted *loxP* sites as evidenced by DA75357. These results indicated that there was a different problem in the construct, besides the orientation of the *loxP* sites.

### 5.2. Transformation with a weak Cre-expressing plasmid causes BFP expression to either decrease or increase depending on presence of terminators in construct

The two experiments that had been previously performed produced opposite results. The experiments being (1) an assembly PCR that inverted the remaining *loxP* site in the already

recombined DA32004 strain. And (2) an assembly PCR that inverted both *loxP* sites in DA32004, creating DA75357, which was subsequently recombined. Both (1) and (2) should produce genetically identical constructs and were therefore expected to have identical expression of BFP in the resulting strains.
Instead (1) produced a strong BFP signal while (2) did not. Due to this discrepancy of results, DA75355 and DA75357 (**Fig. 6A, 6B**) produced in experiment (2) were both transformed with the same Cre-expressing plasmid pSB1A2 (DA21802) used in (1). MACS showed that DA75355, which had had its terminators removed even before addition of Cre, exhibited a slight decrease in its BFP expression (**Fig. 6C**). In contrast, DA75357, retaining its terminators before recombination, increased its BFP expression upon recombination (**Fig. 6D**).

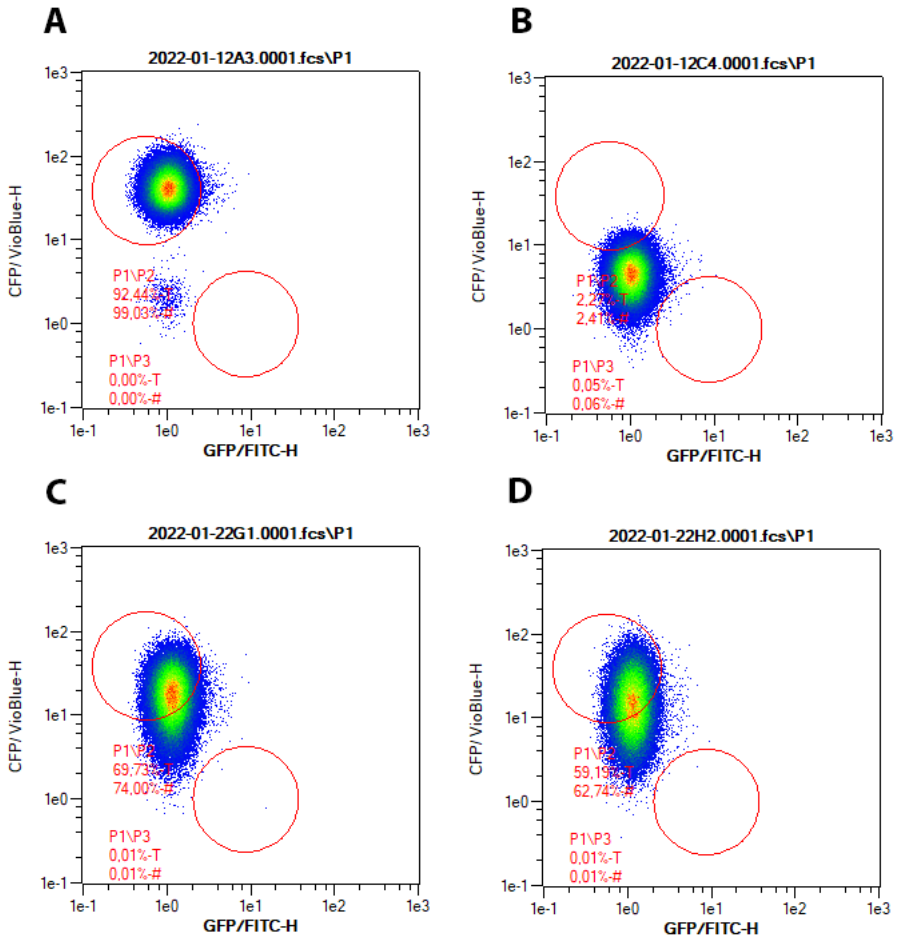


**Figure 6. DA75355 and DA75357 containing the BFP lox-switch with inverted *loxP* sites exhibits decreased and increased BFP expression, respectively, when transformed with the weak Cre-expressing pSB1A2 plasmid.** 1 µL of overnight cell culture was diluted in 200 µL of filtered PBS and incubated at room temperature for a minimum of 20 minutes before analysis using MACS. A minimum of 100,000 cells were counted. The Y-axis shows BFP expression. (A) DA75355, lacking terminators, is a strong expressor of BFP before addition of Cre. (B) DA75357, retaining the terminators, is a weak expressor of BFP before addition of Cre. (C, D) Transformation with pSB1A2 (DA21802), a weak Cre-expressor, slightly decreases BFP expression in DA75355 and increases BFP expression in DA75357.

This result indicated that there is an important difference in recombination as facilitated by either the pSB4S15 or pSB1A2 plasmid. There were no important differences identified between the plasmids at first glance, as they were both just simple expression vectors for Cre. Worthy of note however is that pSB1A2, unlike pSB4S15, was found lacking a dedicated promoter for Cre. What pSB1A2 carried instead was a promoter present outside of its multiple

cloning site with previously documented readthrough into the cloning site where Cre is present. This readthrough should be able to produce a small amount of Cre which then was able to cause recombination.
At this point it seemed likely that the opposite effects on BFP expression caused by the two different plasmids was dependent on the different concentrations of Cre produced in the cell. Also that the elusive error source was not necessarily only caused by a sequence present in the BFP lox-switch, but depended on interacting with another element such as Cre.

### 5.3. BFP-lox switch with an extended promoter region exhibits BFP expression as intended

Transformations with plasmids expressing Cre either weakly or strongly gave us an indication that the presence of Cre was somehow interfering with expression of BFP in a concentration-dependent manner. Addition of a strong Cre-expressor was able to shut down BFP expression in both the terminator-lacking (DA75355) and terminator-retaining (DA75357) strains. At the same time addition of a weak Cre-expressor decreased BFP when the terminators were prematurely absent, and increased BFP when they were not. This should not be the case since Cre's sole function is to catalyze a recombination event that removes a pair of terminators in the BFP lox-switch. This recombination event was expected to increase BFP expression in the non-recombined strain, and have no effect in the already recombined strain.
We noted that the distance between the promoter and the first *loxP* site was very short and hypothesized that binding of Cre to the remaining *loxP* site might interfere with RNA-polymerase binding to the promoter. To ascertain whether the Cre recombinase could be transiently binding the *loxP* site remaining after recombination and blocking the transcription machinery necessary for BFP expression, a new assembly PCR was designed. The previously produced BFP lox-switch with inverted *loxP* sites present in DA75355 and DA75357 was amplified and later produced in two separate segments. The segments put together constituted the whole construct with the addition of 26 non-coding basepairs between the J23-101 promoter and the first *loxP* site. The two fragments were combined and inserted into a new DA24100 strain lacking the BFP lox-switch, giving rise to DA75405 and DA75409. DA75355 served as the parent strain for DA75405, and DA75357 as the parent for DA75409.
DA75405 contained no terminators and DA75409 still retained its original terminators. Both strains were transformed with the same Cre-expressing plasmids used previously; pSB4S15 (DA27758) and pSB1A2 (DA21802). BFP expression was analysed in MACS (**Figure 7**). It was shown that DA75405 did not notably change its BFP expression when either of the Cre-expressing plasmids was introduced (**Fig. 7C, 7E**). Dissimilarly, DA75409 increased its expression of BFP when either Cre-expressing plasmid was introduced (**Fig. 7D, 7F**). Both plasmids expressing either a low or high amount of Cre gave rise to a BFP signal with similar strength in DA75409. Both DA75405 and DA75409 were sequenced to verify the extension of the promoter region which had been performed without any adverse effects.

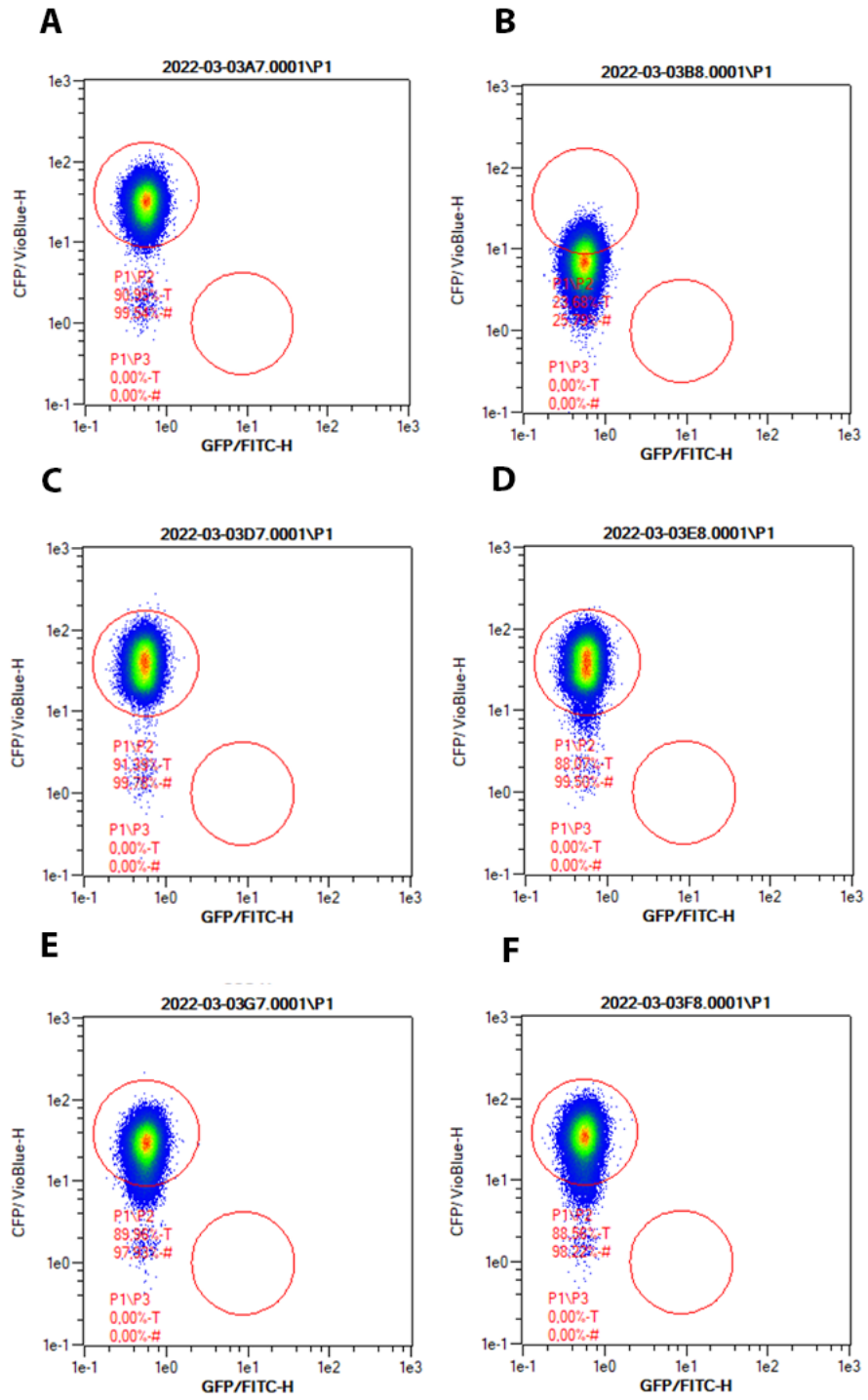


**Figure 7. DA75405 and DA75409 containing inverted *loxP* sites and an extended promoter region exhibit either no change or increased BFP expression, respectively, when transformed with a weak or strong Cre-expressing plasmid.** 1 µL of overnight cell culture was diluted in 200 µL of filtered PBS and incubated at room temperature for a minimum of 20 minutes before being analysed using MACS. A minimum of 100,000 cells were counted. The Y-axis shows BFP expression. (A) DA75405 is a strong expressor of BFP before addition of Cre. (B) DA75409 is a weak expressor of BFP before addition of Cre. (C, D) Transformation with pSB4S15 (DA27758), a strong Cre-expressor, did not affect BFP expression in DA75405 and increased BFP expression in DA75409. (E, F) Transformation with pSB1A2 (DA21802), a weak Cre-expressor, did not affect BFP expression in DA75405 and increased BFP expression in DA75409.

This result indicates that the BFP lox-switch retaining the terminators as present in DA75409 now works as intended. As designed, BFP expression is at a low level in the strain before

addition of Cre, and turns on in the presence of a strong as well as a weak Cre-expressing plasmid.

### 5.4. Exchanging the J23-101 promoter increases BFP signal gain after recombination

It is of importance that we are able to tell with certainty when a recombination event has occurred, as evidenced by BFP expression turning on. Recombination events are detected by measuring BFP expression caused by the reporter gene *mTagBFP2*. BFP expression is initially inhibited and turns on in response to Cre removing two terminators present in the construct, then allowing for the promoter present in the construct to drive expression of *mTagBFP2*. However, the best promoter for our construct would be one that produces a minimal amount of BFP leakage before recombination and a strong signal once the terminators are removed.

In order to calibrate BFP expression before and after addition of Cre, the J23-101 promoter present in our construct was replaced with other promoters from the J23 family using overlapping primers and assembly PCR. The following promoters from the J23 family were selected: 105, 106, 110, 114, 115, and 118. Of these, 106 & 118 were expected to be relatively strong, 105 & 110 slightly weak, and 114 & 115 to be very weak inducers of transcription.

The BFP lox-switch was amplified in two fragments from DA75405 and DA75409. The fragments were later combined in a second PCR similarly to what was done to extend the promoter region in the construct. Lambda red recombineering into DA24100, lacking the BFP lox-switch, was performed separately for all the selected promoters that were successfully produced using assembly PCR. This includes 105, 106, 114, and 118 for DA75405 as well as 105, 110, 114, 115, and 118 for DA75409. Transformation of the strains derived from DA75409 with a strong (pSB4S15) and weak (pSB1A2) Cre-expressing plasmid was also performed.

In the constructs produced with DA75409 as parental strain, MACS showed a comparable level of BFP expression before recombination for the promoters: 105, 110, 114, 115, and 118 as compared to the original J23-101 (**Figure 8**). Also similar to the original DA75409 construct containing J23-101, addition of a low or high amount of Cre via plasmid produced a nearly identical level of BFP expression after recombination (for each individual promoter). Although a bigger net increase in BFP expression after recombination was attained with 105, 110, 114, and 118 as compared to J23-101. 115 produced a similar profile to J23-101, possibly with a lower BFP signal before recombination.

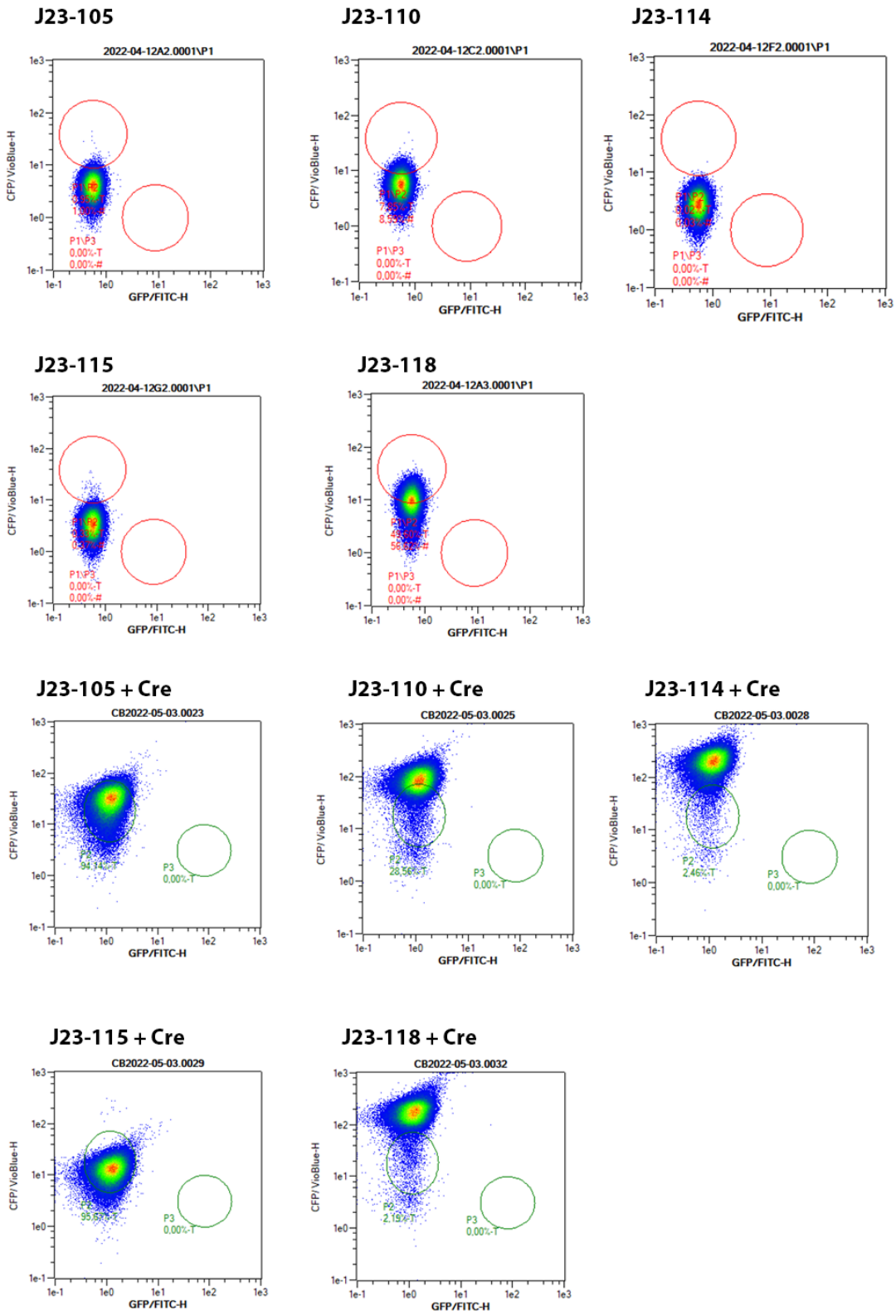


**Figure 8. Different J23-promoters inducing BFP in DA75409 exhibit a bigger net increase in signal after recombination as compared to J23-101.** 1 µL of overnight cell culture was diluted in 200 µL of filtered PBS and incubated at room temperature for a minimum of 20 minutes before being analysed using MACS. A minimum of 100,000 cells were counted. The Y-axis shows BFP expression.

Observing strains derived from DA75405 (**Figure 9**), lacking the terminators in the construct, 106 & 118 produced a strong BFP signal as expected. 105 produced a weaker signal,

comparable to an unrecombined strain retaining the terminators. 114 in a contradictory fashion produced both BFP-negative and BFP-positive colonies.

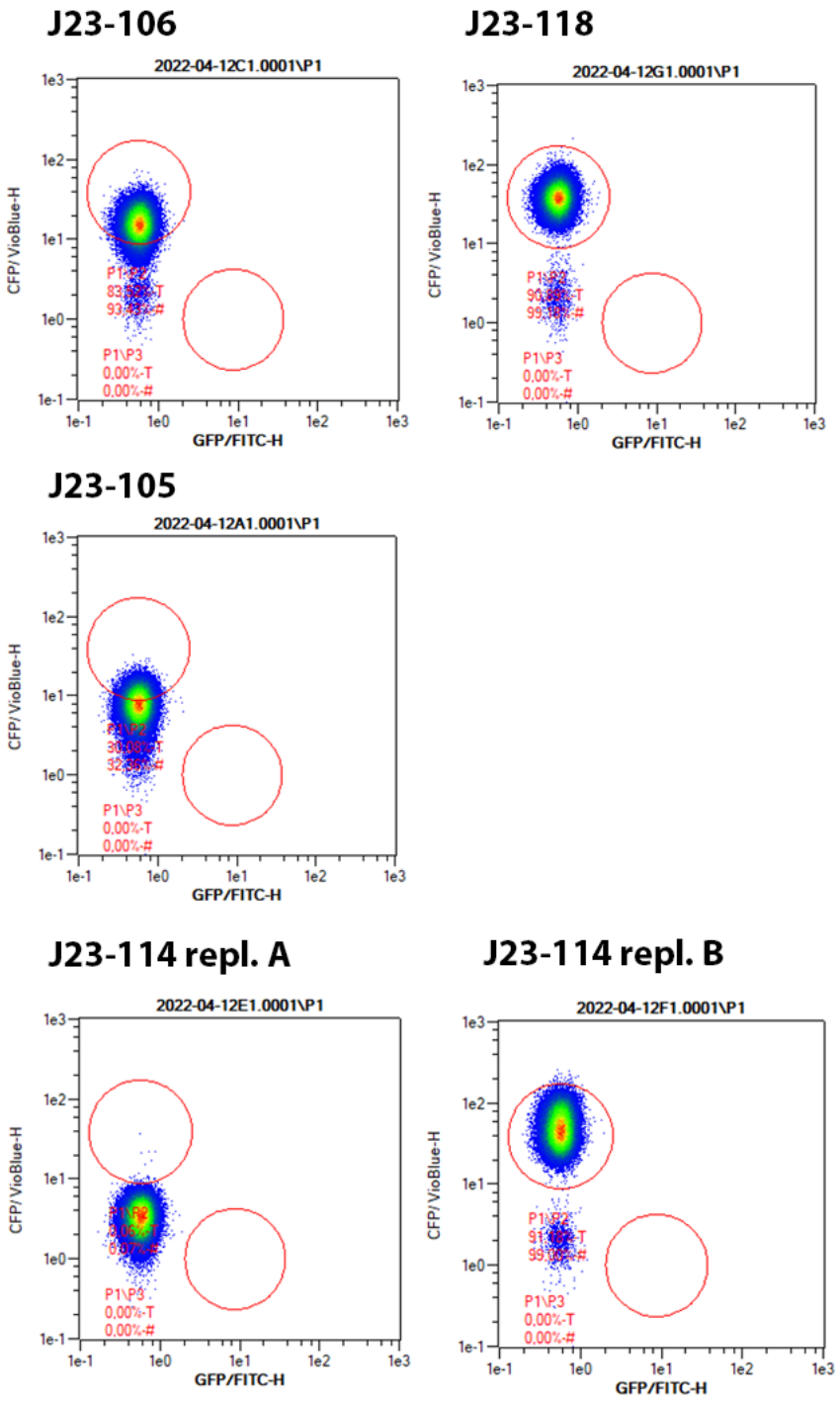


**Figure 9. Different J23-promoters inducing BFP in DA75405, lacking terminators, exhibit varying levels of BFP.** 1 µL of overnight cell culture was diluted in 200 µL of filtered PBS and incubated at room temperature for a minimum of 20 minutes before being analysed using MACS. A minimum of 100,000 cells were counted. The Y-axis shows BFP expression. repl. = (biological) "Replicate"

The promoters that seemed best suited to replace J23-101 in DA75409 were 105, 110, and 114. This due to showing only a low level of BFP expression before recombination, and a higher level of BFP expression after recombination relative to J23-101. Surprisingly, our current

strains with 114 produced biological replicates that were strongly BFP-positive even before addition of Cre, both in DA75405 and DA75409 (data not shown for DA75409).
Sequencing validated the sequences for all promoters that were successfully produced, with the exception of 114. In DA75409, 114 was shown to contain two sets of neighboring, erroneous basepairs in two different parts of the J23-114 promoter. In DA75405, one of the biological replicates had two erroneous basepairs while the other had the intended sequence. The presence of these mutations in the J23-114 promoter might explain the premature expression of BFP in one of the DA75409 replicates. As well as the lack of BFP expression in one of the DA75405 replicates.

### 5.5. Transformation and conjugation with YFP-expressing plasmids does not produce YFP as expected

Our model depends not only on detecting BFP expressed from the bacterial chromosome but also yellow fluorescent protein (YFP) expressed from the conjugative plasmid. The combination of a BFP signal which turns on in presence of the plasmid and a constitutionally active YFP on the plasmid will allow us to distinguish between plasmid donors and recipients and calculate conjugation frequencies accurately. Furthermore, by having a signal present on the plasmid it will be possible to discern whether cells retain the plasmid and for how long.
To investigate how MACS would measure BFP and YFP expressed at the same time in individual cells, DA75405 and DA75409 were transformed with a variety of YFP-expressing plasmids: pDA_VaC::yfp (DA46327), pSB3S5-SYFP2_opt-B1006 (DA25827), and pSC101_YFP110_Cre (no DA #). However, analysis in MACS showed no YFP expression despite transformations being carried out according to standard protocol and producing selectable colonies (**Figure 10**). Transformation with the YFP- and Cre-expressing plasmid pSC101_YFP110_Cre caused recombination to occur in DA75409 as evidenced by BFP expression turning on (**Fig. 10B**). However, no measurable amount of YFP could be detected. This result was unexpected since the plasmids used were known to produce YFP-positive cells when transformed into other MG1655 bacterial strains.

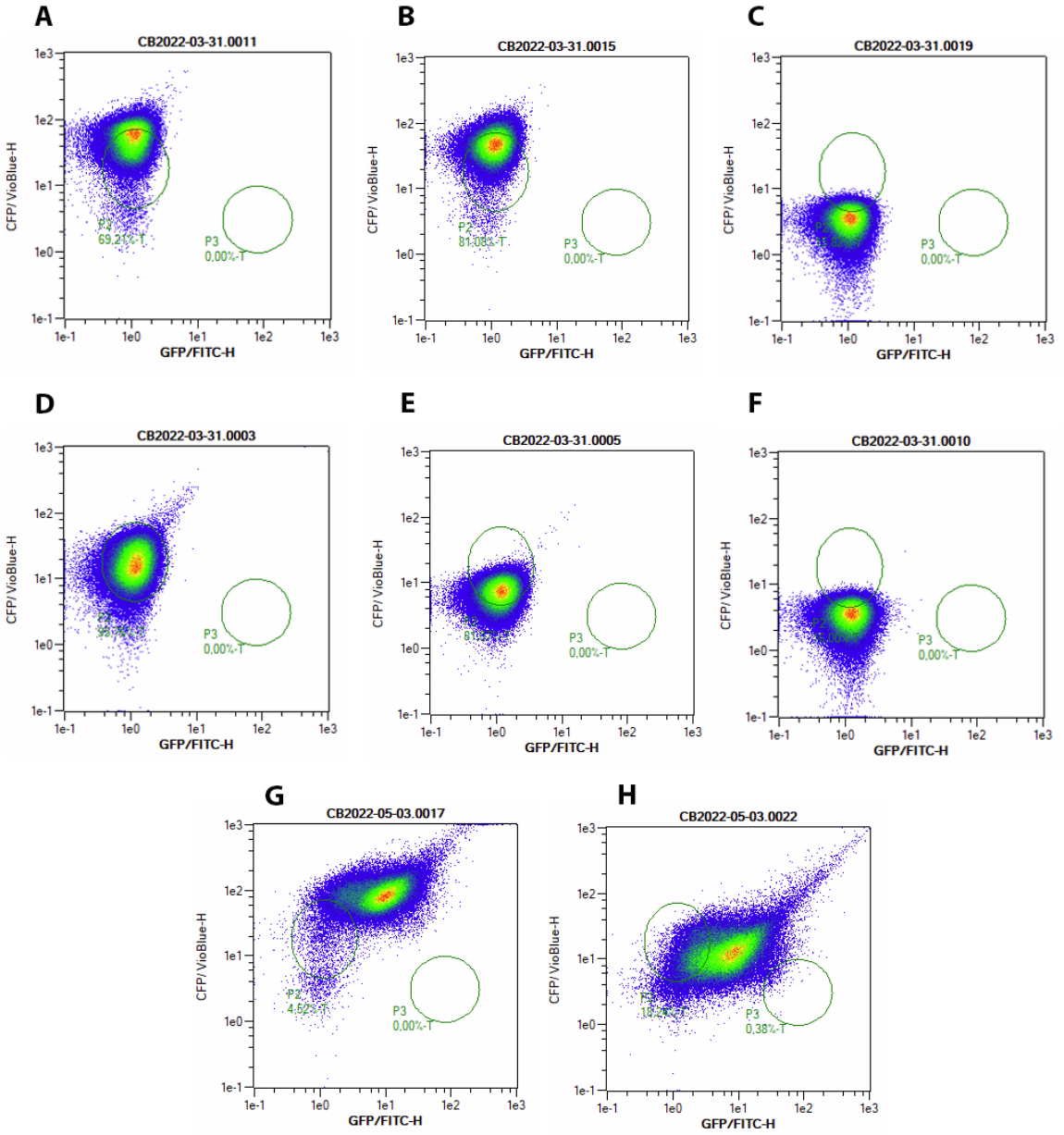


**Figure 10. DA75405, DA75409, and DA4201 do not express YFP when transformed with YFP-expressing plasmids.** 1 µL of overnight cell culture was diluted in 200 µL of filtered PBS and incubated at room temperature for a minimum of 20 minutes before being analysed using MACS. A minimum of 100,000 cells were counted. The Y-axis shows BFP expression, the X-axis YFP expression. (A, B, C) DA75405, DA75409, and DA4201 respectively, do not express YFP when transformed with the Cre- and YFP-expressing plasmid pSC101_YFP110_Cre. (D, E, F) DA75405, DA75409, and DA4201 respectively do not express YFP when transformed with an YFP-expressing pDA_VaC::yfp plasmid (DA46327). (G, H) DA75405 and DA75409, respectively, produce a moderate level of YFP as well as a high variance in signal when conjugated with the YFP-expressing plasmid pJNEG01 from DA25015.

To rule out a false negative in the MACS machine itself, DA28200 which has a chromosomal, constitutively active *SYFP2* induced by a J23-101 promoter was measured and shown to be strongly YFP-positive (data not shown).

DA75405 and DA75409 are another potential source of error as it cannot be ruled out that the BFP lox-switch interferes with YFP expression via an unknown mechanism. To investigate this, DA4201 which is an MG1655 strain lacking the BFP lox-switch was transformed with the Cre- and YFP-expressing plasmid pSC101_YFP110_Cre, as well as the YFP-expressing

plasmids from DA25827 and DA26550. However, all transformations failed to produce any YFP as measured by MACS (**Figure 10**) (data not shown for DA25827 and DA26550).
DA75405 and DA75409 were also conjugated in two steps using the DAP-dependent strain DA32305 as a middleman. This produced a DA75405 and DA75409 strain carrying the YFP-expressing pJNEG01 plasmid (DA25015) which gave rise to a moderate YFP signal but also a very high variance of both BFP and YFP between cells, measured by MACS (**Fig. 10G, 10H**). These experiments show that there is some unknown issue that stops us from inducing and/or measuring YFP expressed from a plasmid. This occurs not only in our strains (DA75405, DA75409) but also in a standard MG1655 strain lacking the BFP lox-switch (DA4201).

# 6. Discussion

This study aimed to construct and evaluate a conjugation tracking system. The system would consist of two parts: (1) A conjugative plasmid constitutively expressing both YFP and the Cre recombinase. (2) A floxed chromosomal BFP present in a bacterium capable of receiving plasmids via conjugation. Significant progress has been made on the floxed chromosomal BFP construct to the point that BFP expression now behaves as was originally intended. The working construct has also been used as a backbone for a variety of strains with different J23-promoters. These new strains can be used for further iterations, aimed at producing the best possible contrast of BFP before and after recombination.
However, additional work is required to produce a YFP- and Cre-expressing conjugative plasmid that can be used in conjunction with the chromosomally floxed BFP. Also to adapt both parts of the system to clinical strains and plasmids that are of interest to the original question posed: Does the presence of subinhibitory levels of antibiotics affect conjugational frequency?

## 6.1. The BFP lox-switch

During inversion of the loxP sites it was initially unknown what caused the very strong and premature expression of BFP in two of the recombineered strains (DA75355 & DA75359, referenced as “55” and “59”), or the leaky expression in the third strain (DA75357, referenced as “57”). The premature expression of BFP in 55 and 59 was hypothesized to be caused by a premature recombination event, which was confirmed by sequencing. It has to be the case that the premature recombination event not catalyzed by Cre happens during LRR at the earliest. Otherwise LRR would not have been able to produce even a single strain with both *loxP* sites still present in the construct together with the terminators. Why this recombination occurred is still unknown: The only procedural difference between samples during LRR was the amount of purified BFP lox-switch DNA that was added to DA24100. In our case, the samples with the lowest and highest volumes of added DNA (55 and 59, respectively) produced a premature recombination whereas the medium volume (57) did not.
It is worrying that premature recombination has been shown to occur in our construct. Not only because it is unexpected but also because it could prove disruptive depending on the frequency of occurrence, which might vary depending on the receiving bacteria. LRR is necessary to move the BFP-lox switch to different bacteria of interest to be assayed. Individual verification would have to be carried out to check that BFP is not expressed prematurely. Not to suggest that the LRR protocol has been optimized for our purposes. Without doubt there is room for tuning and standardizing e.g. how the BFP lox-switch is prepared and how much DNA is used during electroporation of different bacteria.
To address BFP expression in our strains: 57 exhibited a slight leakage in expression despite the presence of the T1 and T7 terminators. Two theoretically competent terminators, albeit with varying degrees of efficiency, that were in frame with and should be able to inhibit the expression of *mTagBFP2*. It is not improbable that the J23-101 promoter is too strong for the

purposes of this conjugation tracking system and that the leaky expression is a result of the T1 & T7 terminators not being able to inhibit it fully. The potency or "strength" of promoters are determined on assays with many variable conditions which cannot be corrected for when adapting them to different uses, hence why work was performed to construct strains with different promoters from the J23-family specifically for our construct. The aspiration was to find a promoter in the J23 family which produced the best possible contrast between non-recombined and recombined strains and thus a reliable signal with which to measure conjugation events.
Just like the modification and rescue of the BFP lox-switch at large, this has undoubtedly been a success. There is already a series of BFP-lox switches with different J23 promoters that show a promising pattern of very little BFP expression before addition of Cre, and very strong expression of BFP after recombination.

### 6.2. Cre-expressing plasmids

As was shown, transformation with different types of Cre-expressing plasmids produced both unexpected and conflicting results with regards to BFP expression: In our original construct, the strongly Cre-expressing plasmid pSB4S15 decreased BFP expression regardless if the terminators in the BFP lox-switch were present (DA75357) or absent (DA75355 and DA75359). This produced the exact opposite effect from the one intended: Introduction of Cre is supposed to cause a recombination event that removes the T1 and T7 terminators from the construct, which leaves the J23-101 promoter free to initiate transcription of *mTagBFP2* and induce a BFP signal.
Another Cre-expressing plasmid that was used, pSB1A2, had an effect where a strain lacking terminators, DA75355, had its BFP signal reduced. The opposite was shown for a strain retaining the terminators, DA75357, which saw an increase in BFP expression. pSB1A2 lacks a dedicated promoter for Cre but nonetheless produces a modicum of Cre, likely due to readthrough into the cloning site from a promoter present upstream in the plasmid. It stands to reason that Cre had an unexpected and concentration-dependent effect on BFP expression in our bacteria containing the BFP lox-switch.
A hypothesis explaining both the decrease and increase in BFP expression is that Cre transiently binds the *loxP* site remaining after recombination. The result is a steric hindrance which blocks the transcription machinery necessary to initiate transcription of *mTagBFP2*. Different effects in strains either lacking terminators (DA75355) or retaining the terminators (DA75357) can be understood by thinking about the net effect of Cre when added to the system. If recombination has occurred prematurely and the terminators are already gone, Cre will only hinder BFP expression by binding the remaining *loxP* site. If the terminators are still present, Cre will cause a recombination event removing the terminators and increasing BFP expression. In the latter case, inhibition of BFP expression will also occur but is outweighed by the removal of the terminators which strongly induces BFP expression.
In the case of a strong Cre-expressing plasmid (pSB4S15), the overabundance of Cre tilts the scales in favor of inhibition. This causes BFP expression to drop to an identical level in both strains, regardless if the terminators had already been removed by a premature recombination event. In contrast, a weak Cre-expressing plasmid (pSB1A2) inhibits BFP expression somewhat due to transient binding in the strain lacking terminators. The strain retaining its terminators sees a net increase due to the recombination event and removal of terminators having a stronger effect than the inhibition caused by Cre binding the *loxP* site. Cre is a widely known and used method to induce controlled changes in a genome, especially changes detected using a reporter gene. That Cre would be able to inhibit transcription of a reporter gene by binding the *loxP* site remaining after recombination, which at this time remains a theory, was an unexpected finding.

Thankfully, with the modifications we carried out, the BFP lox-switch now functions as intended. Both in the presence of a low and high amount of Cre as produced by two different plasmids.

### 6.3. YFP-expressing plasmids

At the moment of writing, it is not known why we were unsuccessful in producing YFP-positive cells by introducing a plasmid expressing YFP. This has been the case both in our strains containing the BFP lox-switch (DA75405, DA75409) and an essentially unmodified MG1655 strain (DA4201). A variety of YFP-expressing plasmids were transformed as well as a conjugation experiment performed. Probably hundreds of transformations have been performed previously in the lab, using the same or similar plasmids, and were able to produce YFP-positive cells using the same methods and strains derived from MG1655 or similar *E. coli* strains.
As evidenced by DA28200, the J23-101 promoter is able to drive YFP, at the very least of the *SYFP2* variety. Transformation with a plasmid expressing both Cre and YFP (pSC101_YFP110_Cre) showed that a recombination event occurred in DA75409, evidenced by BFP expression increasing. This indicates successful transcription and translation of Cre from the same plasmid carrying YFP after insertion into DA75409. Cre and YFP are both driven by the J23 family of promoters which have shown to be effective in our strains, at least for driving expression of *mTagBFP2*.

### 6.4. Potential applications of the tracking system and further development

It could be of interest to observe conjugational frequencies not only in the presence of singular antibiotics at low concentrations, but also multiple antibiotics at varying concentrations. It has been observed in *E. coli* that the combination of kanamycin and streptomycin can increase the proliferation of plasmids in a population while neither antibiotic has this effect alone (26). These findings could be verified, and other combinations of antibiotics tested, using our system.
Bacteria in the environment often inhabit complex communities known as biofilms that can form heterogenous and stratified structures with different cell types, gene expression, and phenotypic behavior dependent on cues from the environment (35). Of interest to us is how plasmid dissemination functions in a biofilm, or rather a model based on biofilms. Using fluorescence microscopy and our tracking system, the movement of plasmids via conjugation could potentially be measured and analysed in a time-lapse. Conclusions could then be drawn how e.g. participating bacterial species, general biofilm structure, and environmental pressures (antibiotics) affect the “when and where” of plasmid dissemination.
In this study, kanamycin and spectinomycin were used for selection of the chromosomal BFP lox-switch and the plasmid cassette, respectively. They were chosen as they were good candidates for developing the conjugation tracking system and could be selected for in our MG1655 strains. Alas, clinically relevant bacteria and plasmids are likely to be resistant to at least a few antibiotics of those used to treat infections, if not a plurality of antibiotics in the case of the MDR plasmids. To use the system in these pathogens we must have access to a small library of our tracking system with different resistance genes for selection after recombineering and transformation. The library of strains should be relatively easy and inexpensive to produce using already established methods, mainly assembly PCR, but is still work that remains to be done. Constructing this library could be the next step in further improving the system’s usability, even if priority should be given to the Cre- and YFP-expressing plasmid.

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## 8. Supplementary Information

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

| Temp (°C) | Time | Cycles |
|---|---|---|
| **98** | 5´ | x1 |
| **98** | 30" | x27 |
| **60** | 30" | |
| **72** | 1´30" | |
| **72** | 7´ | x1 |
| **4** | ∞ | |

| **Phusion™ High-Fidelity DNA Polymerase** | |
|---|---|
| **Reagent** | Volume |
| **$dH_2O$** | 23.7 µL |
| **2mM dNTP** | 5 µL |
| **5µM F** | 5 µL |
| **5µM R** | 5 µL |
| **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 |
|---|---|---|
| **98** | 5´ | x1 |
| **98** | 30" | x2 |
| **64** | 30" | |
| **72** | 2´30" | |
| **98** | 30" | x2 |
| **62** | 30" | |
| **72** | 2´30" | |
| **98** | 30" | x2 |
| **60** | 30" | |
| **72** | 2´ | |
| **98** | 30" | x7 |
| **59** | 30" | |
| **72** | 2´ | |
| **98** | 30" | x23 |
| **72** | 2´ | |
| **72** | 7´ | x1 |
| **4** | ∞ | |

| **Phusion™ High-Fidelity DNA Polymerase** | |
|---|---|
| **Reagent** | Volume |
| **$dH_2O$** | 22.2 µL |
| **2mM dNTP** | 5 µL |
| **5µM F** | 5 µL |
| **5µM R** | 5 µL |
| **5x HF Phusion Buffer** | 10 µL |
| **DMSO (99%)** | 1.5 µL |
| **Phusion polymerase** | 0.3 µL |
| **DNA template** | 1 µL |

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

| Primer | Sequence (5'to 3') | Comment |
|---|---|---|
| **BFP_ampl_R** | ATGAAGCTGTACATGGAAGG | Sequencing BFP switch. |
| **BFP_Inv_F_Term** | CGTATAGCATACATTATACGAA GTTATTCACACTGGCTCACCTTC | Inversion of *loxP* site. |
| **BFP_Inv_R1_Term** | AAAAAATTAGCGCAAGAAGAC | Inversion of *loxP* site. |
| **BFP_Inv_R2_Term** | CGTATAATGTATGCTATACGAA GTTATAAAAAATTAGCGCAAGA AGAC | Inversion of *loxP* site. Same binding part as BFP_Inv_R1_Term, with addition of tail for inversion. |
| **BFP_Inv_R1_AP** | GACCGCCGTCGGCCGGAA | Inversion of *loxP* site. Very high melting temperature. |
| **BFP_Inv_R2_AP** | CGTATAATGTATGCTATACGAA GTTATGACCGCCGTCGGCCGGA A | Inversion of *loxP* site. Very high melting temperature. Same binding part as BFP_Inv_R1_AP, with addition of tail for inversion. |
| **ColiGalKYFPLinR** | GTTTGCGCGCAGTCAGCGATATCC ATTTTCGCGAATCCGGAGTGTAAG AACGCCTTTGAGTGAGCTGATA | Amplification of BFP switch. 40 base pair homology for insertion into *galK*. |
| **ColiGalK_SF** | GGTTATGAAATGCTGGCAGA | Amplification of BFP switch. |
| **ColiGalK_SR** | GACTACCATCCCTGCGTTGT | Amplification of BFP switch. Sequencing BFP switch. |

| | | |
|---|---|---|
| **Distpromo_loxP F** | ACGCGTTCTAGGTACTGTAGTACGAGCTACTAGAGATAACTTCGTA | Extension of BFP lox-switch between J23-101 promoter and remaining *loxP* site. |
| **Distpromo_loxP R** | CTACAGTACCTAGAACGCGTACCGTAGCTAGCATAATACCTAGGAC | Extension of BFP lox-switch between J23-101 promoter and remaining *loxP* site. |
| **galK_scrn_F** | TACGCAAAGTTAACAGTCG | Amplification of BFP switch. |
| **galK_scrn_R** | CAGAGGCGGATAAAAGTG | Amplification of BFP switch. Sequencing BFP switch. |
| **Lox_inversion_F** | CGTATAGCATACATTATACGAAGTTATACTAGAGAAAGAGGAGAAATACTA | Inversion of *loxP* site. |
| **Lox_inversion_R** | CGTATAATGTATGCTATACGAAGTTATCTCTAGTAGCTAGCATAATACC | Inversion of *loxP* site. |
| **OL_galK_scrn_F** | ACGGGCGGAAAGTAAAGTC | Screening inserts in *galK*. |
| **OL_galK_scrn_R** | AATGGCGAAGAGAATCAAC | Screening inserts in *galK*. |
| **prol_J23-105_F** | TACGGCTAGCTCAGTCCTAGGTACTATGCTAGCTACGGTACGCGTTC | Exchange of the J23-101 promoter. |
| **prol_J23-105_R** | TAGTACCTAGGACTGAGCTAGCCGTAAACTCTAGAAGCGGCCGCGA | Exchange of the J23-101 promoter. |
| **prol_J23-106_F** | TACGGCTAGCTCAGTCCTAGGTATAGTGCTAGCTACGGTACGCGTTC | Exchange of the J23-101 promoter. |
| **prol_J23-106_R** | CTATACCTAGGACTGAGCTAGCCGTAAACTCTAGAAGCGGCCGCGA | Exchange of the J23-101 promoter. |
| **prol_J23-110_F** | TACGGCTAGCTCAGTCCTAGGTACAATGCTAGCTACGGTACGCGTTC | Exchange of the J23-101 promoter. |
| **prol_J23-110_R** | TTGTACCTAGGACTGAGCTAGCCGTAAACTCTAGAAGCGGCCGCGA | Exchange of the J23-101 promoter. |
| **prol_J23-114_F** | TATGGCTAGCTCAGTCCTAGGTACAATGCTAGCTACGGTACGCGTTC | Exchange of the J23-101 promoter. |
| **prol_J23-114_R** | TTGTACCTAGGACTGAGCTAGCCATAAACTCTAGAAGCGGCCGCGA | Exchange of the J23-101 promoter. |

| | | |
|---|---|---|
| **prol_J23-115_F** | TATAGCTAGCTCAGCCCTTGGT ACAATGCTAGCTACGGTACGCG TTC | Exchange of the J23-101 promoter. |
| **prol_J23-115_R** | TTGTACCAAGGGCTGAGCTAGC TATAAACTCTAGAAGCGGCCGC GA | Exchange of the J23-101 promoter. |
| **prol_J23-118_F** | GACGGCTAGCTCAGTCCTAGGT ATTGTGCTAGCTACGGTACGCG TTC | Exchange of the J23-101 promoter. |
| **prol_J23-118_R** | CAATACCTAGGACTGAGCTAGC CGTCAACTCTAGAAGCGGCCGC GA | Exchange of the J23-101 promoter. |

| | | |
|---|---|---|
| **pEL_galK_Eco_F** | TCAGCGACAGCTTGCTGTACGGCA GGCACCAGCTCTTCCGCACACAAC CACACCACAC | Amplification of BFP switch. 40 base pair homology for insertion into *galK*. |
| **VF_2** | TGCCACCTGACGTCTAAGAA | Sequencing BFP switch. |

**Supplementary notes 1**

CLC data, sequencing data, MACS results, and protocols used can be found here:
*https://drive.google.com/drive/folders/1n7c5Bk_Wk_eYIiWZWk1jaO6XJjKHKAh7?usp=sharing*