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UPTEC X 22027

Examensarbete 30 hp
Juni 2022

Trade-offs in CRISPR Immunity against Mobile Genetic Elements

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Civilingenjörsprogrammet i molekylär bioteknik

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Abstract

The prokaryotic adaptive immune system CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) is a defense mechanism that helps to protect the prokaryotic cell from invading mobile genetic elements. This project was performed at Uppsala University and served to answer whether the expression of Cascade, which is part of the CRISPR defense system, will have a negative effect on the cell that expresses it and to also determine whether the CRISPR defense system is effective enough to stop the spread of a conjugative plasmid. A microfluidic system was used in order to perform the experiments and images were taken with the help of fluorescent microscopy.

Three different donor strains from *E.coli* were used. These strains had their own version of the RP4 conjugative plasmid which had the ability to infect recipient *E.coli* cells with said plasmid. The recipient cells had the ability to express the CRISPR system in order to defend themselves from the plasmid and CRISPR was also inducible with the help of IPTG. The different versions of the RP4 conjugative plasmid had different amounts of spacer targets that Cascade, the recognition complex in the CRISPR system, could recognize.

When the recipient cells were induced and had a known target sequence of the plasmid they were able to defend themselves and keep the number of transconjugant cells low. When the recipient cells did not know the target the amount of transconjugant cells were higher. It was also noted that when the cells were induced inside the microfluidic PDMS chip they had a slower generation time.

It was also noted that recipient cells had begun to die towards the end of the microfluidic experiments when the cells were induced. This raised the question as to whether the CRISPR defense system was targeting itself as well as the RP4 conjugative plasmid.

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Är Bakteriers Immunförsvar Svaret på Antibiotikaresistens?

Populärvetenskaplig sammanfattning

Johanna Cederblad

Vår omgivning är full av olika mikroorganismer som ibland kan vara bra för oss och som ibland kan göra oss sjuka, så kallade patogener. Vi människor kan särskilt under vinterhalvåret, ofta vara hängiga och förkylda på grund av de mikroorganismer som vi stöter på. Vi blir sjuka för att våra kroppar ännu inte vet hur de ska försvara sig mot de organismer som orsakar sjukdom hos oss människor och som vi stöter på för första gången. Vi har dock ett immunförsvar som finns där för att hantera sådant som gör oss sjuka och som också hjälper oss att komma ihåg patogenen så att vi inte ska bli sjuka av samma sak en gång till.

Bakterier är en grupp mikroorganism som vi människor inte kan se med blotta ögat, men dessa små organismer kan också bli sjuka på grund av att de har blivit smittade av något som de inte heller vet hur de ska försvara sig emot. Ändå så kan bakterier ofta klarar sig ganska så bra. Det är nämligen så att något så litet som en bakterie också måste kunna försvara sig mot sådant som gör den sjuk. Därför så har bakterier också ett slags immunförsvar som kommer ihåg det som har gjort den sjuk tidigare och som hjälper till och skyddar bakterien mot att bli sjuk igen. Det här immunförsvaret för bakterier kallas för CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats).

Man skulle kunna jämför bakteriens immunförsvar med en person som letar efter en annan individ i en folkmassa. Om personen som letar aldrig tidigare har sett personen som den letar efter så är det näst intill omöjligt att hitta rätt person utan att be om hjälp eller interagera med andra individer i folkhavet. Om personen som letar däremot tidigare har träffat personen eller sett denne på bild så kan personen hitta rätt; betydligt enklare och snabbare än tidigare. På samma sätt kan man tänka sig att bakteriers immunförsvar fungerar. Om bakterien inte har sett det som försöker komma in i och smitta cellen sedan tidigare, så kommer inte försvaret att reagera då det inte kommer att känna igen det som något farligt. Om bakterien däremot har stött på en bit av inkräktaren tidigare, som i exemplet med personen som letar och får se en bild på den som den letar efter, så kan bakterien känna igen inkräktaren så fort den kommer in i cellen och agera på en gång.

Denna igenkänning hos bakterien är det som jag har tittat på och undersökt i mitt projekt. Är det så att kostnaden som läggs på igenkänningen gör så att bakterien blir långsammare i jämförelse med sina grannar, samt är det så att bakterien kan upptäcka allt som kan göra den sjuk eller hinner den inte med?

Om bakterien skulle kunna känna igen sådant som är farligt för oss människor men som inte vi kan se och snabbare än vad vi kan upptäcka det, så skulle vi kunna använda bakterier för att försvara oss mot inkräktare med hjälp av bakteriernas egna immunförsvar. CRISPR skulle kunna vara en nyckel för att bota infektioner hos oss människor. Huruvida vi kommer att kunna samspela med bakterier på det här sättet är något som återstår att se.

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Abbreviations

CRISPR Clustered Regularly Interspaced Short Palindromic Repeats

crRNA CRISPR RNA

dsDNA double-stranded DNA

E.coli *Escherichia coli*

HGT Horizontal Gene Transfer

IPTG Isopropyl β-D-1-thiogalactopyranoside

LOC Lab-on-Chip

MGE Mobile Genetic Element

O/N culture Overnight

oriC Origin of Replication

oriT Origin of Transfer

PAM Protospacer Adjacent Motif

PDMS polydimethylsiloxane

SSB Single-Stranded Binding protein

ssDNA single-stranded DNA

T4CP type IV Coupling Protein

T4SS type IV Secretion System

TF Transcription Factor

1 Introduction

How is it possible to image the defense system in bacteria? This project aims to monitor and measure the defense mechanism CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) that serves to defend the bacterial cell from invading mobile genetic elements (MGE) (Marraffini & Sontheimer 2008). MGEs can be transferred through conjugative plasmids, and one type of gene that is frequently spread this way is antibiotic resistant genes (Kim *et al.* 2021). With the help of microfluidics, it is possible to monitor the adaptive immune system of bacterial cells and measure what is going on inside them (Whitesides 2006).

The aim of this project is to monitor the conjugation between donor strains and recipient strains on a single-cell analysis level. This will be done in order to investigate whether it is possible to follow conjugation, if the CRISPR defense system is negatively affecting the cell that expresses it and if the CRISPR defense system is fast enough to prevent the spread of the invading MGE.

1.1 Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)

Clustered Regularly Interspaced Short Palindromic Repeats, or for short CRISPR, is widely known both in the scientific world and among common people. CRISPR is an adaptive defense system in prokaryotic cells that defends the cell from invading mobile genetic elements (Marraffini & Sontheimer 2008). In the year 2020, the Nobel Prize in Chemistry was awarded to Emmanuelle Charpentier and Jennifer Doudna who both had made extensive contributions to science due to the CRISPR system (Nobel Prize 2020). They together changed the native CRISPR system so it would be easier to handle and they also managed to recreate the system *in vitro*. Today CRISPR is used in many different applications around the world such as gene therapy to restore the vision of individuals with Leber congenital amaurosis type 10 (Maeder *et al.* 2019), sickle cell anemia (Akinsheye *et al.* 2011) and cancer treatments (Baylis & McLeod 2017). These are only some of the many usages for the famous gene-editing scissor (Uddin *et al.* 2020).

Despite the many benign possibilities that CRISPR provides it also opens up ethically questionable stands. The ethical dilemma was thoroughly raised when He Jiankui, a Chinese scientist, performed gene-editing on human embryos in the hope of giving the future individuals HIV resistance (Greely 2019). Since the gene-editing performed on the embryos could be inherited by the affected individual's future children and the edit could give rise to unknown effects that will only be known once the individual has grown older. The gene-edit gave rise to a man-made probable gene defect and an alternation in the genome of the individuals which they had not asked for.

However, the incredible tool that has been given with CRISPR might weigh heavier than the ethical dilemma. In the end, only time will tell what role, good or bad, that this new tool will play.

1.2 CRISPR and the Fitness of the Bacterial Cell

The CRISPR systems original function is to act as an adaptive defense mechanism created by prokaryotes in order to defend themselves from invading mobile genetic elements (MGE), such as viruses or conjugative plasmids (Marraffini & Sontheimer 2008, Barrangou 2015). A piece of the invading MGE that comes from the virus or plasmid is saved in a CRISPR array. This array consists of spacers which are part of earlier invading MGEs, and repeated sequences that function as borders between the different MGEs. These spacers are incorporated into the loci of the CRISPR array. When the CRISPR gene is transcribed, all of the spacers and repeats are transcribed as pre-crRNA. After a maturation step, they are turned into CRISPR RNA (crRNA). The crRNA is then used to find the specific target sequence of the invading MGE. The Cas endonuclease can then with the help of the crRNA cut the targeted nucleic acid sequence. In order for the Cas endonuclease to cut the target sequence that is complementary to the crRNA, it needs to have a Protospacer Adjacent Motif (PAM) sequence as well (Synthego 2022). The PAM sequence is usually between 2-6 base pairs long and is located 3-4 base pairs downstream of where the Cas endonuclease makes the cut in the target sequence. The PAM sequence is specific for the organism's specific CRISPR system.

The CRISPR system is a versatile tool when it comes to biotechnological applications due to the easy way that a target can be chosen and the independent workflow of the system itself. If a specific sequence is wished to be altered then it is possible to use tools that are available in the lab and computer programs in order to design the specific site. This simple approach makes it easy for most scientists to use it as a tool to edit genes in different organisms. There are currently six different types of CRISPR systems: I, II, III, IV, V and VI (Liu *et al.* 2021). These six types of CRISPR systems have subtypes within them. In the scope of this master thesis, I have worked with the I-E CRISPR system found in *Escherichia coli* (*E.coli*).

CRISPR has the ability to act as a defense mechanism for bacterial cells that have the system in it. However, it has been shown that the search time for the Cascade complex used in the 1-E CRISPR system takes around 1.5 hours in order for it to find its target (Vink *et al.* 2020) and that it takes around 6 hours for the famous CRISPR-Cas9 in the type two system, to find its target (Jones *et al.* 2017). Therefore a hypothesis has been made that a large amount of Cascade is needed in order to reach an effective defense in the cell. The Cascade complex is made up of the Cas1, Cas2, Cas5, Cas6, Cas7, Cas8e and Cas11 proteins (Xue & Sashital 2019). The Cas3 is the CRISPR protein which is recruited by the Cascade and makes the cut of the target sequence of the MGE.

CasA is a component of the CRISPR complex that was used in this project. CasA (also known as Cas8e) is part of the protein complex that binds to the target sequence in the type I-E CRISPR system (Xue & Sashital 2019). CasA recognizes the PAM sequence and recruits Cas3 which then makes the cut in the target sequence.

1.3 Antibiotic Resistance

A global health threat that has become more severe due to human mishandling of antibiotics is antibiotic resistance. Due to the overuse of this class of drug, a selective pressure has been

created for bacteria to resist it (Pelgrift & Friedman 2013). Between the years 2000 and 2010, the use of antibiotics increased by 35% worldwide (Van Boeckel *et al.* 2014). The way an antibiotic resistant gene can be acquired is either through conjugation between bacteria, transformation of elements that are accessible around the bacteria or transduction by viruses (Vrancianu *et al.* 2020), so there are several ways for antibiotic resistant genes to be obtained by the cell. The antibiotic resistance is not the same for all antibiotics and it is not as abundant in all parts of the world. For example, Methicillin-resistance is somewhere at 12.11% for *Staphylococcus aureus* and cephalosporins resistance in *E.coli* is at 36%. It is also estimated that it will not be possible to treat nearly 60% of those that have multi-drug resistant tuberculosis (WHO 2021). The need to find an alternative tool to battle antibiotic resistance is apparent and CRISPR could be possible to use since it is the bacteria's natural defense against MGEs (Getino & de la Cruz 2018).

1.4 Conjugative Plasmids

The way that information and new genes can be shared between bacteria is often through horizontal gene transfer (HGT) (Shen *et al.* 2022). One type of genes that are often shared this way is antimicrobial resistance and one component that is especially used in HGT is conjugative plasmids. Conjugative plasmids are plasmids that themselves carry the amount of genetic information needed in order to be transferred into another cell (Raleigh & Low 2013). They are, in another word, self-transmissible. The content of a conjugative plasmid can be beneficial for the cell that receives it or simply just a burden and an unnecessary cost. The so-called accessory genes, that can be transferred between cells due to conjugative plasmids are among some, antibiotic resistance genes. These genes are not necessary for the bacteria's ability to survive and can be seen as a bonus gene that can be used to enhance survival in certain situations. It might also be regarded as a redundant gene that is lost after a couple of generations if the bacteria is not exposed to the specific antibiotic (Kim *et al.* 2021). In order for a plasmid to be self-transmissible it typically has: oriT (origin of transfer), a type IV secretion system (T4SS), a type IV coupling protein (T4CP) and a relaxase (Raleigh & Low 2013). The relaxase creates a nick in the plasmid which is going to be transferred, then single-stranded DNA (ssDNA) of that plasmid is brought to the T4CP at the cell membrane where it is brought through the T4SS into the recipient cell, there it is replicated into a double-stranded DNA (dsDNA) plasmid and can now be used in the new owner of the plasmid (Getino & de la Cruz 2018). Even though antimicrobial resistant genes can be beneficial for the bacteria, it has started to become a global health threat to humans and animals (Kim *et al.* 2021). It has also been shown that conjugative plasmids require a higher fitness cost on the recipient bacterial cells that have just obtained the plasmid (San Millan & MacLean 2017); and that a newly transconjugated bacteria that have just obtained the conjugative plasmid will have a slower generation time than the bacteria that are already used to the plasmid (Prensky *et al.* 2021).

1.5 Microfluidics

The need to perform experiments on a smaller scale have given rise to the so-called Lab-on-Chip (LOC) approach that is possible with a microfluidics system (Whitesides 2006). Due to the micrometer size of the channels, other obstacles arise as compared to experiments performed

on a larger scale. Liquids do not tend to mix in microfluidics since they experience laminar flow (Bragheri *et al.* 2020). Therefore, if the need to mix arises then certain components are needed in the channels, such as sharp turns or herringbone structures, which will allow turbulence to happen. Important factors that need to be considered are: Reynolds number (Re), Péclets number (Pe) and the capillary number (Ca) (Squires & Quake 2005). In the experiments during this project, mixing has been made prior to the microfluidic run, therefore the problem with mixing was eliminated. Pluronics was also used which is a component that is needed in order to make the flow easier through the microfluidics system and to mainly prevent cells from sticking to each other, clogging the channels in the chip. A thorough description of microfluidics and its parameters can be found in Squires & Quake (2005).

Depending on the use of the microfluidic chip, different designs can be made. The design is often made in CAD or a similar program and then transferred onto a mold with the help of a soft-lithography method where a negative or positive photoresist can be made in order to make the master mold (Zhang & Hoshino 2019). The chip is later made with polydimethylsiloxane (PDMS), which cures into a soft gel-chip that is later bonded to a substrate, often a glass slip. The bonding is performed by plasma treating both the PDMS and the glass slip and then quickly assembling them (Figure 1) (Bragheri *et al.* 2020). The design of the chip is made in such a way that it allows the imaging of the cells to be only of the cells that are in their exponentially growing phase and over a long period of time. The flow through the chip helps to push out cells that are approaching their stationary phase since these cells are not as important to the project.

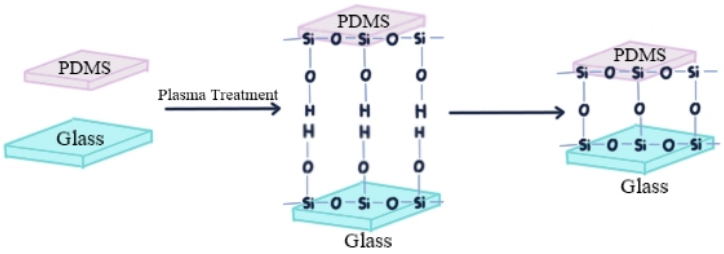


Figure 1: The image is showing a schematic drawing of what happens when the PDMS and the glass slip are bonded together. Prior to the treatment, the surface has methyl (-CH_3) groups which are converted into reactive silanol (-SiOH) groups. The bonding of the two surfaces results in covalent bonds that prevent the chip from leaking during microfluidic runs.

1.6 The Partitioning System in Plasmids

Partitioning systems are frequently used as a labeling tool in order to monitor targets in the cell. For low copy number plasmids, like the P1 plasmid, there is a partitioning system incorporated which ensures that the daughter cells both will have the plasmid after replication has occurred (Nordström & Austin 1989), the so-called P1 *par* operon (Hao & Yarmolinsky 2002). The partitioning system found in P1 plasmids consists of the *parS* locus and the ParB and the ParA proteins. The *parA* locus is located upstreams from the *parB* locus which in turn is located upstream of the *parS* locus. When ParB is in its protein form it binds to the *parS* and forms a complex. ParA can then be recruited to the complex which will form a fiber-like structure (Hao & Yarmolinsky 2002). ParA is an ATPase that forms an ATP gradient in the cell. The *parS* is often located near the origin of replication (oriC), so when the ParB-*parS* complex is formed during replication, the two daughter chromosomes are pushed away from each other

ensuring that each daughter cell will have a copy of the plasmid (Badrinarayanan *et al.* 2015). The ParB-*parS* complex moves towards the ParA-ATP gradient at one end of the cell. This makes it possible for the ParB-*parS* complex to pull the plasmid to one end of the cell, ensuring that one copy will be in each of the daughter cells. In this project, the P1 partitioning system is used as a marker for one of the targets in the bacterial cell, namely the RP4 conjugative plasmid.

1.7 The Project and its Goals

The goal with the project was to determine whether the amount of CRISPR expressed in a bacterial cell will affect the growth rate in the cell and to also see how efficient the defense system is against conjugative plasmids.

In order to investigate if the amount of CRISPR being expressed affect the fitness of the cell, different variants of the RP4 conjugative plasmid were used. See Figure 2 for a schematic image of how the three different versions of the RP4 conjugative plasmid look. The image is not the full CRISPR array and only serves to show the difference between the versions. The first variant had all of the complementary sequences for the spacers in the CRISPR array incorporated in the plasmid. This ensures that the Cascades would definitely find the plasmid as a target. The second variant had one complementary sequence of the spacer in the CRISPR array incorporated in the plasmid. This variant is the one that is the closest to the natural occurring CRISPR arrays since these usually have one target from one invader or MGE. The last variant had no complementary sequence to the spacer in the CRISPR array. This variant could be regarded as the negative control and aims to visualize the population without a defense against the conjugative plasmid.

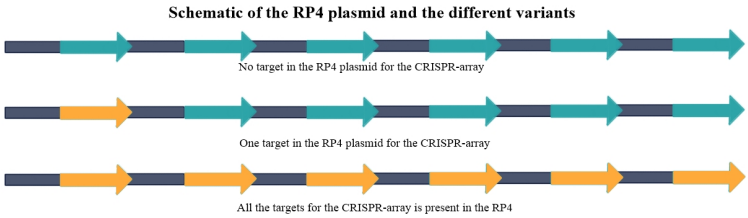


Figure 2: A schematic image of the three different variants of the RP4 conjugative plasmid found in the three different donor strains: EL3336 (top), EL3337 (middle) and EL3338 (bottom). The blue arrows represent a random spacer sequence while the yellow arrow represent a known sequence and target for Cascade. The complete spacer sequence is not present in the schematic image, only the first six spacer sequences. The EL3336 has only random spacer sequences in the RP4 plasmid, so no targets for the Cascade. Strain EL3337 has one target for the Cascade in the RP4 plasmid and EL3338 has all 18 targets for the Cascade in the RP4 plasmid.

The RP4 conjugative plasmid has had the pMT1parS sequence inserted. This sequence allows ParB to bind to it. However, *parA* is missing from the cassette, therefore no fiber will be made between ParA and ParB. Instead ParB has a fluorescent marker, mCherry, fused to it. This marker will fluoresce when it is excited with a wavelength at 594 nm (Lambert 2022a). When the *parS* locus is present mCherry-ParB will accumulate and form a bright spot, indicating where the plasmid is located in the cell. The mCherry-ParB can therefore be used as a tag on the *parS* site on the RP4 plasmid (Figure 3). With this, it is possible to see which cells that have the conjugative plasmid and to also see how the plasmid is spreading in the population.

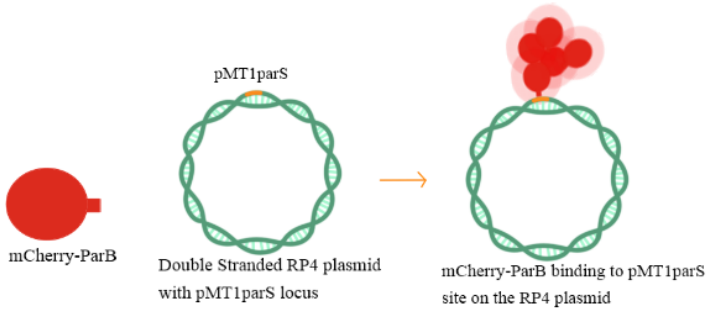


Figure 3: The image shows mCherry-ParB and how it targets the ParS *site on the RP4 plasmid and how the binding is possible to see when the fluorophore is excited and emits light. The mCherry-ParB only targets the* parS *when the plasmid is in the shape of dsDNA.*

The project also uses SSB (Single-Stranded Binding protein) which has a fluorescent YFP-marker attached to it, namely Venus. The protein SSB has a ssDNA binding domain and an amphipathic C-terminal (Shen *et al.* 2022). In *E.coli*, it is in the shape of a stable homo-tetramer. The SSB protein is important when it comes to the replication, repair and recombination of DNA in bacterial cells. It plays a stabilizing part in the handling and preservation of ssDNA during the processing of DNA (Savvides *et al.* 2004).

The SSB-Venus binds to single-stranded DNA and fluoresces when it is excited by wavelengths at 514 nm (Figure 4) (Lambert 2022c). When the conjugated plasmid first enters a new cell it is in the shape of linear ssDNA (Shen *et al.* 2022). The SSB-Venus will therefore bind to the linear ssDNA, it will then be possible to trace the conjugative plasmid as it enters a new cell. Once the plasmid has been replicated into dsDNA the SSB-Venus will no longer be attached. In a bacterial cell, there are numerous of different linear ssDNA, so the tag will be useful in order to see the amount of ssDNA but it is necessary to look at the other markers as well.

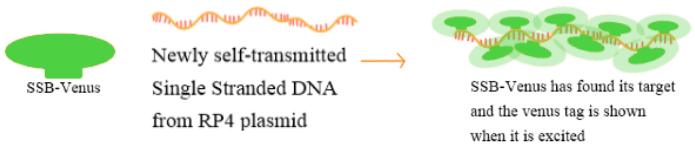


Figure 4: The image shows the SSB-Venus and how it targets ssDNA, here from the newly self-transmitted RP4 plasmid. The conjugative plasmid will be in the form of single-stranded linear DNA when it first enters the cell. The venus fluorophore can also be seen to emit light when it is excited by light from the right wavelength.

The Cascade in the I-E CRISPR system needs Cas3 endonuclease in order to make the cut in the target sequence. Therefore an interesting target would be CasA which is part of the complex that forms Cascade, so a CFP marker named SCFP3A has been fused to the N-terminal of the CasA. The SCFP3A-CasA would then be part of Cascade when it finds its target sequence on the RP4 conjugative plasmid. It would then be possible to see and measure the amount of Cascade that is available in the cell, and that has targeted the RP4 plasmid, when light at wavelength 433 nm excites the fluorophore (Figure 5a) (Lambert 2022b).

Pilot experiments were also performed within the project with one strain that has the CasA fused to the fluorescent marker sYFP2. The conjugated fluorophore, sYFP2, together with CasA

makes it possible to follow Cascade and the possible defense that it provides. The sYFP2 fluorophore is excited with the same wavelength as the Venus fluorophore (Lambert 2022c). The Cascade will bind to the target sequence in the RP4 conjugative plasmid and by exciting the sYFP2 it is possible to see the amount of Cascade and the location of the CRISPR system in the cell. A schematic image of the sYFP2-CasA can be seen in Figure 5b.

(a) SCFP3A fused to CasA, emits light when it is excited at 433 nm. *(b) The sYFP2 fused to CasA excited by light at wavelength 514 nm.*

Figure 5: The images depict the two different fluorophores that are fused to Cascade and how they can look, schematically, when light at the right wavelength is shone on them.

By combining the ability of the conjugative RP4 plasmid, the SSB binding ssDNA, the ParA-ParB-*parS* system and the presence of Cascade, it is possible to choose a target in the *Escherichia coli* cells and perform experiments with a microfluidic system and fluorescent microscopy. The analysis from the experiments can be used to see how the growth rate changes due to the different variants of the RP4 plasmids that are infecting the cells by measuring the amount of Cascade that is being produced in order to defend the cells from the invading MGE.

A possible end goal for this project is to determine whether it is possible to use CRISPR in the fight against antibiotic resistant bacteria. In order to use the gene-editing scissor in that context, a thorough investigation of what CRISPR is capable of is needed. This master thesis project hopes to help in that aspect.

2 Materials and Methods

2.1 Preparation of Cells

Five different strains of *E.coli* were used during this project. Every experiment had one recipient strain and three donor strains; see table 1 for a schematic overlook of the experiments made. The donor strains had their own version of the RP4 conjugative plasmid. Strain EL3336 had only random targets for Cascade on the plasmid, strain EL3337 had one target for Cascade and strain EL3338 had all 18 spacers as targets on the plasmid for Cascade, see Figure 2 for a visualization of the plasmids. The recipient strains were strain EL3354 or EL3203. The EL3354 had an SSB-Venus conjugation in order to detect linear ssDNA present in the cell. Strain EL3203 had the sYFP2 fluorophore fused to Cas8e (also known as CasA), so the occurrence of Cascade could be monitored during the runs. All strains had the mCherry-ParB, so the occurrence of the RP4 conjugative plasmid could be measured. Strains EL3336, EL3337 and EL3338 have Kanamycin resistance, so these cells were prepared with Kanamycin in LB media in an O/N culture the day before an experiment. Strain EL3354 and EL3203 have Kanamycin and Streptomycin resistance and were prepared with these antibiotics in an O/N culture the day before an experiment. The O/N cultures were put into 37°C overnight at 250 rpm shaking. On the day of the experiment, the O/N cultures were diluted in 5ml of M9 glucose RPMI media with 25 μL of O/N culture with a 1:200 dilution. The cells were incubated at 37 °C for another 2-2.5 hours, at 250 rpm shaking, before being loaded onto the chip.

Table 1: A schematic table over the different experiments that were performed and how the donor cells and recipient cells were setup. The + sign is used when the cells are induced and there is an active CRISPR defense and the - sign is used for uninduced cells where CRISPR is not active. The donor cells are on the horizontal label and the recipient is on the vertical axis to the left.

Donor Cells	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Recipient EL3354	-	+	-	+	-	+
Recipient EL3203	-	+	-	+	-	+

The genotypes for the five different strains used in this project can be seen in table 2. The aim of the project was to see how conjugation happened between different strains, so different components were needed. The pKEDR13 plasmid was used in order to control the induction of Cascade. Different fluorophores were also used in order to track specific parts of the conjugation and the cells and different amount of spacer targets were also used for the three donor strains.

Table 2: The different genotypes for the five strains used in the project can be seen in this table. The recipient cells EL3354 and EL3203 both have the plasmid pKEDR13 which makes it possible for the CRISPR system to be induced. The rph+ indicates that a defect that has been present in the E.coli MG1655 is fixed so the strain can survive in minimal media. The :: indicates what has been changed in the sequence (old::new) and the / indicates the plasmid used and the sequence for this.

Cell strain	Genotype
EL3354	MG1655 rph+ lamB::SSB-Venus-FRT gtrA::P58-mCherry-parB-SpR /pCas3 /pKEDR13
EL3203	MG1655 rph+ sYFP2-cas8e gtrA::P58-mCherry:parB-SpR /pKEDR13 pCas3
EL3336	MG1655 rph+ gtrA::P58-mCherry-parB-SpR /RP4-310-randtarget
EL3337	MG1655 rph+ gtrA::P58-mCherry-parB-SpR /RP4-311-target1
EL3338	MG1655 rph+ gtrA::P58-mCherry-parB-SpR /RP4-312-alltarget

2.1.1 Preparing the Media and the Cells

The recipient *E.coli* strains had the plasmid pKEDR13 which is equipped with a *lac* promoter upstream of the *LeuO* locus. LeuO is a transcription factor (TF) that in turn induces the Cascade operon and the expression of this (Arslan *et al.* 2013). Therefore, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was used in order to control the expression of Cascade, so experiments with both induced cells and uninduced cells could be made. Minimal media was prepared with 45mL MQ H_2O with pluronics, 5µL $CaCl_2$, 100µL $MgSO_4$, 2,5mL M9 media with salts, 1mL RPMI and 1mL 20% glucose. For induced cells, 1mM of IPTG was used. The cells were diluted in the minimal media in a 1:200 dilution, 2 hours prior to the experiment.

2.2 Fluorescent Microscopy and Microfluidics

In order to follow what is happening in the *E.coli* cells, different fluorescent tags had earlier been fused to strategic elements. The supervision of the cells was possible due to the use of PDMS chips and by following the progress of the cells with time-laps fluorescent microscopy. Here the workflow is described. The microscope used was a Nikon Eclipse Ti-E (Nikon 2022) and the experiments were prepared with a 20x magnification and performed with a 100x magnification. The microscope was set a 30°C before each experiment. The filter cubes used were: Thorlabs 520E for the Venus tag and Nikon 593A for the mCherry fluorescent tag and Nikon Brightline for the SCFP3A tag. For a full description of the filters see Appendix A.

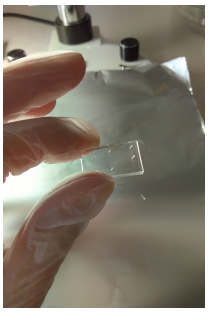
2.2.1 Creating the Microfluidic Chip

In order to perform the fluorescent microscopy experiment with a microfluidics system, a microfluidic chip was needed to be constructed. For this, 45g PDMS was poured into a falcon conical tube with 4.5g (10%) of curing agent. The conical tube was sealed and gently turned, in order to mix, for 30 minutes. The tube was then centrifuged in order to be rid of some of the bubbles in the PDMS mix. For the CRISPR experiments the so-called ”giant chip” would be used with the 1000 nm size in the channels. A glass petri dish was used as a holder for the mold

and the casting of the chip, the inside was covered with aluminum foil. Before using the master mold, dust was gently removed by blowing air with an air pressure gun at an angle towards the master mold. The mold was then placed on top of the aluminum foil with the patterned side up and the PDMS mix was then poured on top of the master mold with the correct dimensions for the channels (Figure 6a). In order to remove more bubbles from the PDMS mix, the glass petri dish with the PDMS was put under vacuum for 20 minutes and then air was let in, this was repeated once. After this, the gel was put into the heating cabinet overnight, at 80°C in order to cure. Once the PDMS had solidified it is possible to gently release the master mold from the aluminum and PDMS and to cut out the relevant chip from the rest of the soft gel (Figure 6b). Holes for the tubings were also made (Figure 6c).

(a) The PDMS before it has been released from the aluminum cast. The master mold is also visible as the dark disc in the bottom.

(b) Here a PDMS chip with the design 1000 nm giant is placed on aluminum foil in order to be prepared for future plasma treatment and bonding.



(c) The PDMS chip is shown after holes have been created to prepare for the insertion of tubes that will be needed during the run.

Figure 6: The images are showing different preparation steps that are needed in order to make a PDMS chip, in order to be able to use it during microfluidic runs.

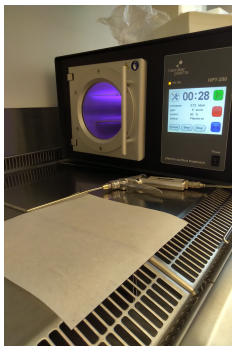
When the polymer has completely solidified (overnight), the chip was cut out with a scalpel and a ruler in order to make clean cuts. It is important to not cut too close to the channels as this can result in leakage during the run. When the chip is free, it is time to puncture holes so the tubings can be connected. The chip was put onto an aluminum foil and put under a microscope in order to see the structure better, a lamp was also directed towards the chip in order to facilitate seeing the structure of the chip. A model was used in order to make holes for the tubes. The structure on the PDMS chip needs to be facing upwards when the holes are made. This is to avoid damaging the channels when reaching the other side of the PDMS with the model. The holes were varied depending on what type of experiment that would be performed. However, a hole for: media inflow, the back channels and the inflow of cells were always made. If dust had settled on the chip it would be removed with tape, no un-gloved hands should touch the structure so gloves should always be worn. The PDMS was bonded onto a clean glass-plate by using plasma treatment.

The plasma treatment of the PDMS chip is performed in order to bond the PDMS to a clean oxidized glass slip which creates a closed compartment for the channels. The bonding to a glass slip also makes it possible to view the channels through a microscope due to the covalent bond that is formed between the two surfaces. The PDMS have surface-exposed methyl groups (-CH_3) which can be made into reactive silanol groups (-SiOH) with the help of plasma treatment (Weibel *et al.* 2007). The plasma treatment can be performed with either oxygen or air. In order to plasma treat a surface, it first needs to be cleaned. Then the surface (the PDMS and the glass slip) is put under vacuum in the plasma machine by depressurizing the compartment. Once vacuum has been reached, air or pure oxygen is pumped into the machine which increases

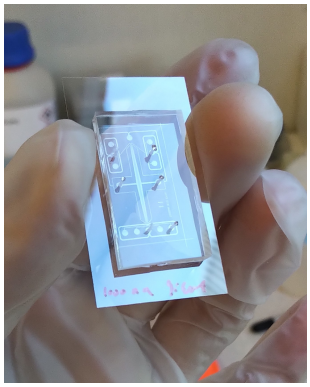
the pressure. The pressure is once again lowered and when it reaches a certain pressure plasma can be formed, which in turn creates a hydrophilic surface. The compartment is once again pressurized and the glass slip and PDMS can be taken out and bonded together.

The first step in bonding the PDMS and the glass slip was to clean it with isopropanol and then dry it by blowing air on it with an air-gun. The chip was then put on the tray for the plasma machine, Henniker Plasma. The glass-plate was also cleaned and dried and put onto the tray as well (Figure 7a). Then the PDMS chip and the glass were plasma treated (Figure 7b), and directly bonded to each other as soon as the plasma treatment was done (Figure 7c). This is to ensure that the formation of the covalent bond is happening. The bonded chip was put into a heating cabinet at 80°C for approximately one hour. Next, the chip was glued to a holder before the run, with only the glass slip being glued.

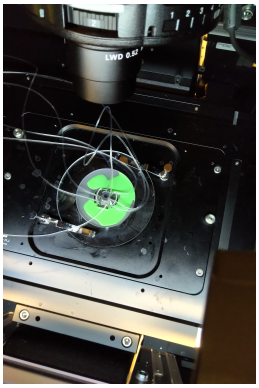
(a) Three PDMS chips together with three glass slips are inserted on a tray into the plasma machine.



(b) How the plasma machine looks once it is performing the plasma treatment.



(c) The result of plasma treating the PDMS chip and bonding it to a clean glass slip.



(d) The PDMS chip inside the microscope with tubes for the pre-made holes have been attached.

Figure 7: Example of how the plasma treatment can look. The PDMS and the glass slips have been cleaned prior to the plasma treatment. The result from bonding a clean chip and glass slip can be seen in image 7c. The placement inside the microscope is also visible with the tubes that connects the media inflow to the chip, allowing media and cells to be applied to the PDMS chip.

2.2.2 Preparing the Microscope and the Chip

The PDMS chip was glued to a holder. The holder fits inside the microscope, and after the glue had dried, the holder with the chip was placed in the right location inside (Figure 7d). Well in advance of the run, the temperature of the Nikon Eclipse Ti2-E microscope was set to 30°C. The increased temperature is set in order to have a pleasant environment for the bacteria. When increasing the temperature, the PDMS will most likely change size which can cause disturbance to the imaging during the run. It is therefore important to increase the temperature before the loading of the chip.

When the holder with the chip had been placed inside the microscope, tubes were connected to the holes that had been made earlier when preparing the chip. The chip was then filled with the media that would be used during the run. Media is applied by increasing the pressure on the pump that is connected to the tube with the media source. After the chip has been filled with media it is possible to load the cells. Here it is important to make sure that there are no air bubbles left in the channels, before adding the cells, since these can cause blockages and hinder the cells from reaching the traps in the chip. The cells were then loaded into the chip

so they ended up in the traps in the middle channel. Once media and cells had been applied in all channels in the chip the program for the run is prepared in MicroManager. Phase images and images for the different fluorescent markers were taken at different time points during the run. The fluorescent images used a light source that would excite the fluorophore at the target in the bacteria. This light was adjusted to different intensities depending on the fluorophore. The mCherry used light at wavelength 594 nm and the measured power density was 21.22 W/cm^2. The Venus tag used light at wavelength 514 nm and had a measured power density of 10.27 W/cm^2. The SCFP3A used light at 433 nm and had a measured power density of 41.09 W/cm^2. The phase images were taken every minute and the images for the experiments with recipient EL3354 had mCherry-ParB taken every ten minutes and the SSB-Venus were taken every other minute. For the experiments with recipient EL3203, the sYFP2-Cas8e was taken every fifteen minutes and the mCherry-ParB was taken every five minutes. The time intervals were chosen so that the conjugation events would be possible to follow. See table 3 for the settings for the run and Appendix B for how the power density, W/cm^2, from the laser was calculated.

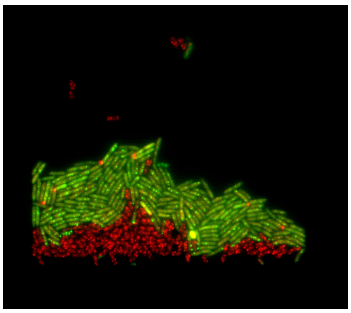
Table 3: The settings for the images that were taken in the fluorescent microscope. The donor cells used for the runs were EL3336, EL3337 and EL3338. One setup had the recipient EL3354 and another setup was with EL3203. The only fluorescent marker that was not present during a microfluidic run was the SCFP3A-CasA. Snap photos were taken of this strain. The program in MicroManager had the following settings.

Strain	Type of image	Intensity	W/cm^2	Image taken	Exposure time
Recipient	Phase	–	–	Every minute	80 ms
	mCherry-ParB	400	21.22	Every 10 minutes	100 ms
EL3354	SSB-Venus	200	10.27	Every 2 minutes	100 ms
	mCherry-ParB	400	21.22	Every 10 minutes	400 ms
EL3354	SSB-Venus	200	10.27	Every 2 minutes	200 ms
	mcherry-ParB	400	10.27	Every 5 minutes	100ms
EL3203	sYFP2-Cas8e	200	21.22	Every 15 minutes	200 ms
EL3404	SCFP3A-CasA	1000	41.09	one image	100 ms

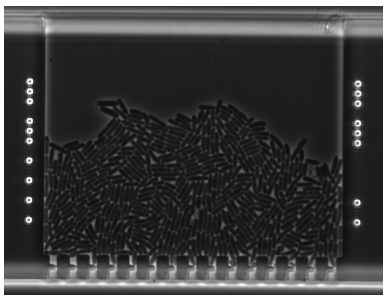
2.2.3 Loading of the Cells

Two different approaches were used when loading the cells for the microfluidic experiments. One had the donor cells loaded first in 20x magnification, then the magnification was switched to 100x and positions for the imaging were chosen. After this the recipient cells (strain EL3354) were loaded and created a layer on top of the donor cells; see Figure 8a for the loading viewed through the fluorescent channels. This approach was used since we wanted to have a clear distinction between the two groups of bacteria and with this also see when the conjugation event occurred and how far the spread could go. The second version for loading, had the donor cells and recipient cells (strain EL3203 or strain EL3354) grown for two hours and after that, the OD was measured. This was to make sure that the cells were in the same growth phase and in an approximately equal amount. The positions on the chip were chosen at a 100x magnification. The next step was to mix equal amounts of cell cultures into a conical tube. The mixed cell cultures were then loaded onto the chip (Figure 8c). The approach to mix the cells prior to

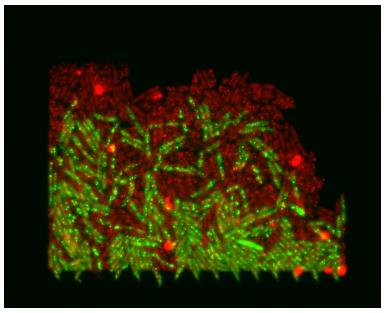
loading them into the chip was used in order to gain an even distribution between the two groups of bacteria (donor and recipient). The mixing also resulted in the cells ending up in random distribution inside the traps which was a desired effect of this method.



(a) The donor cells, seen through the red fluorescent channel, and the recipient cells, seen through the green fluorescent channel, are loaded into a trap. The image is showing an example of how the cells can be arranged right at the beginning of a run.



(b) Here a trap from the giant 1000 nm chip can be seen containing donor cells in the bottom part of the trap, closest to the stripes. The stripes hinder the cells from leaving the trap. On top of the donor cells are the recipient cells.



(c) Here is one example of how the trap can look like when the cells have been mixed prior to loading the cells onto the chip. The red cells are the donor cells and the green are the recipient cells. The image was taken after 45 minutes.

Figure 8: Images of the traps in the giant chip seen both through the phase images and also the fluorescent channels superimposed onto each other, see image 8a and 8c.

Once everything was setup the run could begin and images would be taken at certain intervals, see table 3. An example of what the ”giant trap” look like through the phase images and how the cells are loaded can be seen in Figure 8.

2.3 Growth Curve of Donor and Recipient Cells

In order to see whether the recipient cells had a different generation time from each other and to see if the single-cell imaging showed the same results in bulk experiments, growth curve experiments were performed. O/N cultures with LB and corresponding antibiotics were prepared for the strains of *E.coli*, the day before. M9 glucose media with RPMI was prepared the next day. IPTG was added to half of the media and the other half was left without. The IPTG was added in order to see if the cells grew differently when induced. The induction with IPTG would cause the recipient cells to produce Cascade and activate the CRISPR defense. 20µL of O/N culture was diluted in 20mL of media and incubated at 37°C for 2 hours. After the incubation, 750µL of cells were taken and measured in a UV-Vis spectrophotometry at a wavelength of 600 nm in order to measure OD600. The samples were taken every 30 minutes for 2.5-3 hours, or until OD600 was between 0.3-0.5 for all samples. The result was plotted as the natural logarithm of the OD600 values versus the time of the measurement. The slope that was obtained was the µ parameter which is the growth constant for the organism. From this, the generation time could be obtained together with a confidence interval that originated from the standard error of the mean ($\pm$ SEM), see Appendix C for full calculations.

2.4 Image Analysis

The image analysis serves to determine whether CRISPR is slowing down the cells that are expressing it and if the defense system is effective enough. Therefore, a pipeline called ImAnalysis, which is available from the Elflab, was used in order to analyze the images taken during the experiments. The program FIJI (ImageJ) was used in order to get parameters used in the pipeline, such as the size of the trap that will be analyzed and the location of bar-codes on the traps. FIJI was also used as a sanity check, to confirm whether the images taken are good enough to use and also see how the processed images had turned out.

Scripts were made in MATLAB in order to make the proper analysis, most scripts were premade and readily available in the pipeline. Each data-set with images from the experiments were prepared and parameters were set before running the pipeline. Information such as growth rate and amount of cells that fluoresce could be obtained with this method. Thanks to scripts created during the project, it was also possible to follow the cells and to determine whether the plasmid had been transferred.

Parameters were chosen for the processing of the cells, such as region of interest in the images, how the segmentation should be made and also how the dots from the fluorescent marker should be tracked. After this, the script processCells.m, which is available in the pipeline ImAnalysis, was used in order to process the images so further analysis could be made. The next step was to run the script, plotMeanFluoHistogramsSSBParB.m, which determines the threshold that sorts the cells into groups of donors or recipients, which was then saved to a .txt file. The sorting was based on whether cells had the Venus marker or not, if they did they were regarded as recipients. The .txt file was then used in another script, analyzeRunConjugationSSB.m, that performed the analysis by both looking at the amount of fluorescence from the cells and also classifying the cells based on the fluorescent tags in the cell and if they already had or have obtained the RP4 plasmid. See Appendix D for a full example of the script that was used for induced EL3354 together with EL3336. When the cells had been sorted into donors, recipients or transconjugants, it is possible to further analyze the result. From this, data could be obtained such as growth rate, amount of cells in each group and whether conjugation had happened.

The growth rate for each set of donor cells, recipient cells and transconjugant cells were plotted in a histogram and the mean division time was calculated from this together with a confidence interval that originated from the standard error of the mean ($\pm$ SEM). The confidence interval might not present the full picture since the growth rates of individual bacterial cells are not strictly independent e.g., mother and daughter cells typically have correlated growth rates (Wallden *et al.* 2016). The growth rates are however from individual cells which motivates the use of this method to determine the uncertainty. The mean division time was calculated with the equation:

$$Division\ Time = \frac{log(2)}{Growthrate[\ /min]} \tag{1}$$

The number of cells that fluoresce could be used as a measure to see how the different groups of cells grow and divided in relation to each other. The individual fluorescence for each frame was taken into account. The SSB-Venus and the sYFP2-Cas8e are both specific for the recipient cells EL3354 and EL3203 respectively. From this, it is possible to find and follow the cells in the two groups, donors and recipients, and to determine their mean growth rate and from that

their mean division time. It is also possible to determine if cells from the group ”recipient cells” have become transconjugants (cells that have obtained the plasmid). The mean division time for these three groups for the experiments with recipient EL3354 can be seen in table 4. In the tables mentioned it is also possible to see that variations between each run of experiment and between each day, did occur. An average of the generation time together with uncertainty was calculated, the uncertainty was based on a 95% confidence interval around the mean value. However, an approximate idea of the different cells and the effect that CRISPR has on the system can be observed.

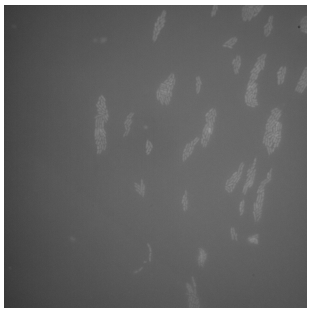
A manual comparison was also made by running the script makeConjugationMovie.m in MATLAB. The script would mark the cells in different categories: donors (red), recipients (green) and transconjugants (yellow). The two fluorescent channels used in the microfluidic experiments, Venus and mCherry, would be merged with each other showing both channels with cells when they emit light. By doing this it is possible to see cells that are classified as transconjugants and have obtained the RP4 conjugative plasmid and to manually compare the classification to the merged image of the fluorescent channels and to see if there are red dots (the RP4 plasmid) in the cells that have been classified as transconjugants.

3 Results

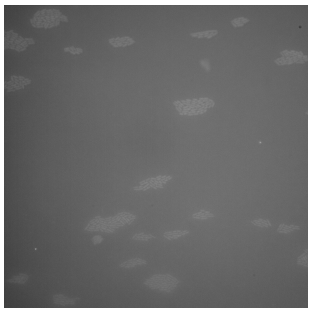
The data showed that the recipient cells that had a Cascade expression mostly had a higher mean growth rate than the donor cells in bulk conditions but that it was not always the case for the cells in the PDMS chip. The data also indicate that when the recipient cells were induced they created Cascade and were also successful in defending the cell when they were aware of the correct target. The median for the generation time for all of the runs can be found in tables 12 and 13 in Appendix C.

3.1 No Apparent Fluorescence was Noted for SCFP3A-CasA

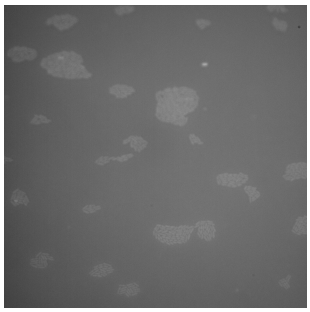
Before the SCFP3A fused to CasA would be used during the microfluidic runs, a test with an agarose pad was made. Images were taken of: a negative control (strain EL3354), positive control (strain EL1679 with SSB-CFP), induced cells with 1mM IPTG (strain EL3404) and uninduced cells (strain EL3404). The intensity was set to 100% (intensity 1000) and the cells were exposed for 100 ms. The positive control is showing spots for the CFP-Cat (Figure 9d). While the induced EL3404 (Figure 9a), and the uninduced EL3404 (Figure 9b), are looking similar to the negative control (Figure 9c). Conclusions from this have lead to the SCFP3A-CasA to not be used as a marker for Cascade during the microfluidic runs. The other two markers have been shown to provide valuable information by themselves. Therefore the project will proceed without this specific fluorescent marker.



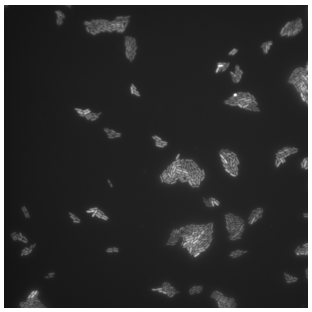
(a) The induced EL3404 with SCFP3A-Cas8E.



(b) The uninduced EL3404 with SCFP3A-Cas8E.



(c) The negative control for the image with strain EL3354.



(d) The positive control with strain EL1916 (CFP-Cat).

Figure 9: The following images illustrate the outcome from using the SCFP3A-CasA and the test images that were taken before the decision to exclude them from the microfluidic runs. It is clear that the induced EL3404, uninduced EL3404 and the negative control with EL3354 look rather similar. The positive control is indeed showing the fluorescent marker. All images have the same settings that were used for the other images. Therefore a decision to not use this fluorophore in this specific setup, was made.

3.2 Induction of Cascade in Recipient Cells Yields Faster Cell Growth in Bulk Conditions

Strain EL3203 was used in growth curve experiments in order to compare them with recipient EL3354. The mean generation time for induced EL3203 was 31.7 ($\pm$0.5) minutes and 33.7 ($\pm$0.5) minutes for uninduced cells. The induction was made with IPTG in order to activate the production of Cascade in the cells. The mean generation time for induced EL3354 was 32.7 ($\pm$1.7) minutes and 34.6 ($\pm$1.2) minutes for uninduced cells. See Appendix C for the generation time, μ and calculations for all experiments. Growth curve experiments with the pre-recipient cells were also made. These showed that the pre-recipient for EL3354 (strain EL3351) had the same mean generation time for both induced and uninduced cells, namely 27.5 ($\pm$1.4 and $\pm$1.3 respectively) minutes. The pre-recipient cells for EL3203 (strain EL3202) had a mean generation time of 29.3 ($\pm$1.4) minutes for the uninduced and 30.1 ($\pm$1.3) minutes for the induced cells. Both the pre-recipient versions had a faster generation time than the recipient cells used in the microfluidic runs. The pre-recipient versions did not have all the components in them that the recipient cells had. Therefore, conclusions could be drawn that when adding the CRISPR system, a plasmid to control CRISPR and fluorescent markers, it slows down the growth rate of the cell. See Appendix C tables 9 and 10 for the generation time and μ values.

3.3 Induction of Cascade did not Affect the Growth Rate of the Donor Cells

Growth curve experiment was made for the three donor strains used in the microfluidic experiments. No great difference was found between the division time for the donor cells since the mean division time for all three is around 29 minutes. The donor cells appear to have a faster generation time than the recipient cells. See appendix C table 11 for all division times and how the calculations were made.

3.4 Tracking Errors During the Image Analysis Led to the Recipient EL3203 to Not be Part of the Results

Due to the segmentation of the cells being of lower quality than what was needed, the tracking of the fluorescent markers during the image analysis was unclear. Therefore, it was not possible to get any valuable data from the experiments with recipient EL3203. A new AI network was trained that would do the segmentation of the cells, but this network was trained towards the end of the project. Due to time constraints in analyzing the data-sets, the data was left out from the results. Therefore another set of experiments with strain EL3354 were made instead.

3.5 Conjugation Assay with Layer Cake Loading Technique

During these set of experiments, the donor cells were loaded first into the traps in the chip. After a layer had been formed, the recipient cells were loaded on top of the donor cells and then the experiment would start. This approach was used since a clear distinction between the two groups was needed. By using this approach it would also be clear to distinguish which cells that would be probable targets for the conjugative plasmid and also see how far the spread of it could reach. The growth rate for all the cells had a cut-off at 0.0001 [/min] in order to include as many cells as possible but not the cells that were dying. The histograms of the growth rates can be found in Appendix E.

3.5.1 The EL3336 Strain (no CRISPR array targets) with Recipient EL3354

The strain EL3336 has no targets for the Cascade. This results in the RP4 conjugative plasmid not being recognized as an intruder by the CRISPR system when it enters a new cell.

A fluorescent microscope microfluidic run was made with EL3336 together with recipient EL3354 with both induced and uninduced cells. The growth rate was plotted in a histogram for the uninduced (Figure 14a) and for the induced cells (Figure 14b). The mean division time for the uninduced cells were: 50.8 minutes (donor), 61.7 minutes (recipient) and 58.1 (transconjugant). The mean division time for the induced cells were: 49 minutes (donor), 60.5 minutes (recipient) and 58.8 minutes (transconjugant). See table 4 for an overview of the mean division time.

3.5.2 The EL3337 Strain (one CRISPR array target) with Recipient EL3354

The strain EL3337 only has one target in the RP4 conjugative plasmid for the Cascade. This version of the plasmid is the one that is closest to the natural occurring CRISPR systems. Due to the plasmid having one spacer target for the Cascade, this RP4 version should be considered as an intruder and should be destroyed by the CRISPR system.

An experiment with a fluorescent microscope with a microfluidic chip was made together with the donor EL3337 and the recipient EL3354 with both induced and uninduced cells. The growth rate was plotted in a histogram for the uninduced (Figure 15a) and the induced (Figure 15b) cells. The mean division time for the uninduced cells were: 45.6 minutes (donor), 53.1 minutes (recipient) and 56.2 minutes (transconjugant). The mean division time for the induced cells were: 49.2 minutes (donor), 71.4 minutes (recipient) and 102.2 (transconjugant). See table 4 for an overview of the mean division time of the cells.

3.5.3 The EL3338 Strain (all 18 CRISPR array targets) with Recipient EL3354

The strain EL3338 has all 18 spacer targets in its RP4 conjugative plasmid, for the Cascade. Therefore this strain should be recognized as an invader by the CRISPR system and the plasmid should be destroyed.

An experiment with fluorescent microscopy together with a microfluidic chip was done with the donor EL3338 and the recipient EL3354 with both induced and uninduced cells. The growth rate was plotted in a histogram for the uninduced (Figure 16a) and the induced (Figure 16b) cells. The mean division time for the uninduced cells were: 54 minutes (donor), 62.6 minutes (recipient) and 62.3 minutes (transconjugant). The mean division time for the induced cells were: 49.4 minutes (donor), 70.6 minutes (recipient) and 47.6 (transconjugant). See table 4 for an overview of the mean division time of the cells.

3.5.4 Induction of Cascade in Recipient Cells During the Microfluidic Experiments Mostly Decreases the Growth Rate

The results of the experiments where the donor cells were first loaded onto the chip and then followed by the recipient cells, are found in this section.

The mean division time for the donor cells (EL3336, EL3337 and EL3338) and the recipient cells (EL3354) can be seen in table 4. In these set of experiments, the same recipient cells were used. The only differences between the runs were whether the recipient cells were induced (marked as +) with IPTG or uninduced (marked as -). The induction activated the CRISPR defense, so induced cells would have produced Cascade while the uninduced cells might not have produced any Cascade. Another difference was the donor cells that were used for the specific run. The same donor cells would be used together with EL3354 once uninduced and once induced. Something that can be noted is that the donor cells appear to have a similar generation time for all of the experiments. Another thing that can be noted from these experiments is that induced EL3354 together with EL3337 (one known target for Cascade) have a profound difference in generation time between the recipient and transconjugant cells, see table 4. The EL3354 seems to be dividing slower in every experiment, compared to donor cells. This is consistent with what was shown from the growth curve experiments where the recipient cells had a slower

generation time in bulk conditions compared to the donor cells. In these experiments, it looks like the transconjugant cells (the recipient cells that have obtained the plasmid) do not always divide slower than the recipient cells that do not have the plasmid. This could however have something to do with how the loading of the cells was made which gave few recipients compared to donors which in turn resulted in giving few transconjugants, see table 5.

Table 4: The mean division time in minutes is presented here for the different donor strains and the recipient/transconjugant strain. - is showing the mean growth for the uninduced cells and the + is showing the mean division time for the induced cells. The mean division time is calculated as log(2)/growth rate. The confidence interval shown in the brackets is based on the standard error of the mean (±SEM).

Cell type	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Induction	-	+	-	+	-	+
Donor	54 (±1.1)	49.4 (±1.5)	45.6 (±1.5)	49.2 (±1.1)	50.8 (±1.3)	49 (±1)
Recipient	62.6 (±3)	70.6 (±14)	53.1 (±2.5)	71.4 (±6.1)	61.7 (±6.6)	60.5 (±7.8)
Transconjugant	62.3 (±8)	47.6 (±8.3)	56.2 (±12.7)	102.2 (±58.4)	58.1 (±11.4)	58.8 (±5.7)

The three different groups of cells appeared in varying amounts. The donors were always in the highest amount followed by the group recipient cells. The transconjugant cells were in the lowest amount for each experiment. It was noted that for the induced experiments the amount of transconjugants is slightly lower when only looking at the amount. The percentage of the donor cells were always higher than 50%, see table 5, while the transconjugant was always lower than 10% of the total amount of cells. The percentage of transconjugant cells were higher for both the induced and uninduced EL3354 together with EL3336. This version had random targets for Cascade, which could be an explanation as to why they are in a higher amount than the rest of the transconjugant cells for the other groups. The experiments with EL3338 had the lowest percentage of transconjugant cells and the experiments with EL3337 were in between EL3336 and EL3338. It is also worth mentioning that the percentage of excluded cells within a group (donor, recipient or transconjugant) is higher when the cells are induced, this is consistent for the transconjugant and recipient cells and almost all of the donor cells except the EL3336 experiments. Here the percentage of excluded cells are slightly higher for the uninduced donor cells than the induced donor cells.

Table 5: The amount of cells for each of the three groups can be seen in the following table, each column represents a microfluidic experiment. Each experiment setup for the EL3354 recipient cell can be seen in the headers. The cells that are counted are the ones that the script in the pipeline regarded as a "good cell". The percentage of the cells in each group can be seen in the brackets after the number. Further pruning of the data was made to exclude cells that had a too slow of a growth rate (slower than 0.0001 [/min]), these cells are shown in the row "Dead cells". The row "Dead cells in group" shows the percentage of the cells within that group that were excluded due to the cell having a growth rate that was below the cut-off. The excluded cells are not part of the total percentage of cells. The row "Amount of cells" only have the cells that are valid under all criteria. The row spacers indicate how many recognized spacer sequences that are present on the RP4 conjugative plasmid found in the donor cells.

Cell type	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Induction	-	+	-	+	-	+
CRISPR targets	18	18	1	1	0	0
Number of cells	26852	10726	16699	18111	19758	21994
Donor	23844	10104	13473	15494	16484	20004
Percentage of total	89%	94.2%	81%	85.6%	83.4%	91%
"Dead" cells	253	129	116	108	181	172
Dead cells in group	1%	1.3%	0.9%	0.7%	1.1%	0.9%
Recipient	2702	600	3043	2406	2643	444
Percentage of total	10%	5.6%	18.2%	13.3%	13.4%	2%
"Dead" cells	50	26	65	90	50	19
Dead cells in group	1.8%	4.3%	2.1%	3.6%	1.9%	4.1%
Transconjugant	306	22	183	211	631	1546
Percentage of total	1.1%	0.2%	1%	1.2%	3.2%	7%
"Dead" cells	8	2	5	9	9	26
Dead cells in group	2.5%	8.3%	2.7%	4%	1.4%	1.7%

3.6 Conjugation Assay with Mixed Cells Loading Technique

In these experiments, the cells were mixed prior to loading them onto the chip. This was due to a need for random distribution to occur in the traps and to also have a more even distribution of the two groups. The settings were in other aspects very similar, one difference however is that older chips were used in most of the experiments. The growth rate was cut-off at 0.0001 [/min] in order to exclude cells that were not growing but still include as many cells as possible. All histograms of the growth rates from these experiments can be found in Appendix E.

3.6.1 The EL3336 Strain (no CRISPR array targets) with Recipient EL3354

The mean division time for the uninduced cells were: 54.9 minutes (donor), 54 minutes (recipient) and 56.6 minutes (transconjugant). The mean division time for the induced donor cells were: 52.2 minutes (donor), 81.5 minutes (recipient) and 135.1 minutes (transconjugant). See Figure 17 in appendix E for the histograms of the growth rate from this experiment.

3.6.2 The EL3337 Strain (one CRISPR array target) with Recipient EL3354

The mean division time for the uninduced were: 57.4 minutes (donor), 55.1 minutes (recipient) and 61.3 minutes (transconjugant). The mean division time for the induced cells were: 53.3 minutes (donor), 74.9 minutes (recipient) and 87.5 minutes (transconjugant). See Figure 18 in appendix E for the histogram of the growth rate from this experiment.

3.6.3 The EL3338 Strain (all 18 CRISPR array targets) with Recipient EL3354

The mean division time for the uninduced cells were: 55.7 minutes (donor), 54.6 minutes (recipient) and 62 minutes (transconjugant). The mean division time for the induced cells were: 54.5 minutes (donor), 87.5 minutes (recipient) and 145.5 minutes (transconjugant). See Figure 19 in appendix E for the histograms of the growth rate from this experiment.

3.6.4 The Mixing of Donors and Recipients Gave a More Even Distribution of the Two Groups of Bacteria

The distribution between donor and recipient cells when using the mixing approach (before the cells were loaded onto the PDMS chip) is much more even, which makes it easier to compare the three groups of cells that emerge from the analysis.

The generation time for the donor cells appears to be similar for all of the experiments. When it comes to the generation time for the different cells, a pattern is beginning to form. The transconjugant cells appear to have a slower generation time. The induced EL3354 together with EL3338 appear to have the slowest generation time for all different microfluidic experiments. It can also be noted that the amount of transconjugant cells are significantly lower for both runs (uninduced and induced) for EL3338 compared to the other two experimental versions. It can also be noted that the amount of transconjugant cells appear to be at a similar amount for the experiments with EL3354 and EL3336, indicating that nothing is present in the cell to stop the spread of the conjugative plasmid and the amount of transconjugant cells to increase. For the experiments with EL3337, it can be noted that the induced experiments is close to half of the amount of transconjugants of the uninduced experiment. The percentage of transconjugants is also significantly lower, indicating that something has happened with the cells and the defense against the RP4 conjugative plasmid which has prevented the spread of it.

In general, it can be noted that the induced cells have a slower generation time than the uninduced cells, when looking at the experimental pairs (EL3354 with EL3338, EL3354 with EL3337 and EL3354 with EL3336), see table 6. Therefore, it seems like there is an extra cost or burden that is put on the bacteria when it is producing Cascade and expressing the CRISPR system.

The transconjugant cells in the induced EL3354 with EL3338 experiment is having the longest generation time at 145.5 minutes. The confidence interval is also the largest which indicates that there is a spread in how the bacteria is handling the synthesis of the RP4 plasmid and the production of Cascade.

Table 6: The mean division time in minutes is presented here for the different donor strains and the recipient/transconjugant strain. - is showing the mean generation time for the uninduced cells and the + is showing the mean generation time for the induced cells. The mean division time is calculated as log(2)/growth rate. The confidence interval shown in the brackets is based on the standard error of the mean (±SEM).

Cell type	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Induction	-	+	-	+	-	+
Donor	55.7 (±3.6)	54.5 (±1.9)	57.4 (±3.2)	53.3(±1.7)	54.9 (±2.4)	52.2(±1.6)
Recipient	54.6 (±1.4)	87.5 (±4.4)	55.1 (±1.5)	74.9(±4.4)	54 (±1.5)	81.5(±6.3)
Transconjugant	62 (±3.6)	145.5 (±33.2)	61.3 (±3.5)	87.5 (±14)	56.5 (±3.7)	135.1(±12.5)

The number of cells for each group in the second run of experiments can be seen in table 7. The transconjugant cells are in the lowest amount regarding representation in percentage. Donors and recipients are not as consistent in the number of cells per group for the runs. This is probably due to the cells being mixed before loading them onto the chip. The groups are, however, more evenly spread than they were during the experiments when they were loaded in layers on top of each other. When the cells are mixed, it can be so that one group of cells can be present in a larger amount during the first run and in a lower amount during the next run. This effect was not regarded as such a big problem since the mixing prior to loading gave a more even distribution of the different groups of cells. This in turn led to the conclusion that this approach is probably the best to use for these types of experiments.

The percentage of excluded cells for each group is higher when the experiment has induced cells, at least for the two groups recipients and transconjugants. This indicates that the cells are not feeling well when they are being induced and therefore result in a higher percentage having a slower growth rate than the cut-off which sorts them into the group of cells that are regarded as ”dead”.

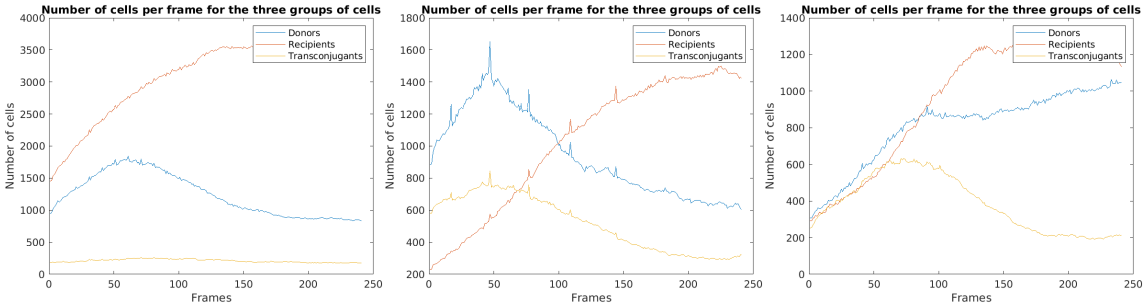
Table 7: The amount of cells for each of the three groups can be seen in the following table, each column represents a microfluidic experiment. Each experimental setup for the EL3354 recipient cell can be seen in the headers. The cells that are counted are the ones that the script in the pipeline regarded as a "good cell". The percentage of the cells in each group can be seen in the brackets after the number. Further pruning of the data was made to exclude cells that had a too slow growth rate (slower than 0.0001 [/min]), these cells are shown in the row "Dead cells". The row "Dead cells in group" shows the percentage of the cells within the specific group that have been excluded due to the growth rate being below the cut-off value. The excluded cells are not part of the total percentage of cells. The row "Amount of cells" only have the cells that are valid under all criteria. The row spacers indicate how many recognized spacer sequences that are present on the RP4 conjugative plasmid found in the donor cells.

Cell type	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Induction	-	+	-	+	-	+
CRISPR targets	18	18	1	1	0	0
Amount of cells	22455	20992	17702	17043	21271	18883
Donor	5198	14543	6645	8526	8413	10892
Percentage of total	23.1%	69.3%	48.2%	50%	39.6%	57.7%
"Dead" cells	79	180	147	160	108	73
Dead cells in group	1.5%	1.2%	2.2%	1.8%	1.3%	0.7%
Recipient	16548	5584	7805	7123	9573	4137
Percentage of total	73.7%	26.6%	44.1%	42.8%	45%	22%
"Dead" cells	122	123	70	143	91	54
Dead cells in group	0.7%	2.2%	0.9%	2%	0.9%	1.3%
Transconjugant	709	865	3252	1394	3285	3854
Percentage of total	3.2%	4.1%	18.4%	8.2%	15.4%	20.4%
"Dead" cells	14	40	48	57	29	125
Dead cells in group	2%	4.4%	1.5%	4%	0.9%	3.1%

3.7 The Conjugation is Visibly Affected by the Induction of the Recipient Cells

It is important to keep track of the amount of cells of each group of bacterial cells (donors, recipients or transconjugants) that are seen in each frame. In Figure 10 the number of cells for each group of cells for the uninduced experiments can be seen. All three graphs are showing fluctuations in the amount of cells that are found in each frame for each group. In Figure 10a (EL3338- all 18 spacer targets) it is possible to see that the transconjugant cells are staying on an even level when compared to the other two graphs. This happens even though the recipient cells are not induced, therefore it is believed that some leakage of Cascade that is looking for the correct target are still available in the cell. Providing a defense against the RP4 conjugative plasmid. In Figure 10b (EL3337- one spacer target) it is possible to see that the transconjugant cells first are increasing and then decreasing as the recipient cells increase. Some leakage of the Cascade with the correct target sequence could also be speculated to be present in the cells. The amount of transconjugants is still more than for the experiment with EL3338 (all spacer targets). In Figure 10c (EL3336- no spacer target) the transconjugant cells can be seen to decrease quite

drastically at frame 100. It is unclear why this is the case, but the amount of transconjugants is at the highest level between the three different experiments. A pattern can be seen which indicates that the amount of spacer targets for the Cascade found in the RP4 plasmid does play an important part in whether the recipient cells can defend themselves from the invading MGE, even though the CRISPR defense is not activated by induction.



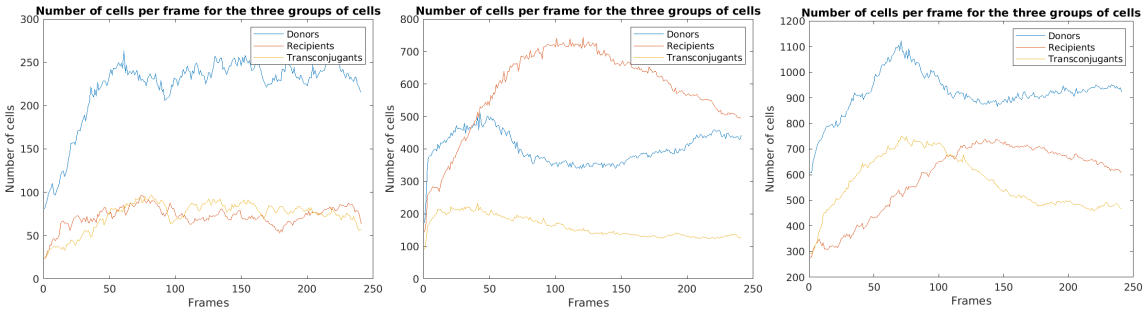
(a) The number of cells in each frame for each group of cells can be seen for the run with uninduced EL3354 together with EL3338.

(b) The number of cells in each frame for each group of cells can be seen for the run with uninduced EL3354 together with EL3337.

(c) The number of cells in each frame for each group of cells can be seen for the run with uninduced EL3354 together with EL3336.

Figure 10: The following images are representing the amount of cells when they are uninduced for the groups: donor(blue), recipient (red) and transconjugant (orange), in every frame. The y-axis represents the amount of cells and the x-axis the frames. There are 241 frames in each run. All three experiments are from experiments with uninduced cells, so the CRISPR defense system is not active. In all three images, the graph for the amount of cells fluctuate for all three groups. No real pattern can be seen. The only observation that seems to be true is that the cells are fluctuating indicating that something is indeed going on inside the traps of the PDMS chip. The experiment with uninduced EL3354 with EL3338 does have an even amount of transconjugant cells when compared to the other two, throughout the entire experiment.

In Figure 11 the amount of cells per frame are plotted, for the induced experiments. This means that the CRISPR defense system is active for the recipient cells. It is possible to see that the experiment with induced EL3354 together with EL3338 does not have an extreme increase in transconjugant cells. The transconjugant cells appear to be at the same level as the recipient cells and do not appear to be decreasing either (Figure 11a). The induced EL3354 together with EL3337 does not seem to have a dramatic increase either, although the recipient cells are increasing. The transconjugant cells seem to be kept at a steady level (Figure 11b). The induced EL3354 together with EL3336 does however have a dramatic increase of transconjugant cells together with the increase of recipient cells. The level of transconjugant cells seem to be decreasing a little but is then leveled at around 500 cells per frame. As mentioned earlier, EL3336 only has random targets for the CRISPR defense system against the RP4 conjugative plasmid that the strain is spreading. The EL3338 has all 18 spacer sequences for the CRISPR array in the RP4 plasmid. Therefore it can be noted that the amount of transconjugant cells seem to be higher when the CRISPR system and the Cascade do not know what their target is.



(a) The number of cells in each frame for each group of cells can be seen for the run with induced EL3354 together with EL3338.

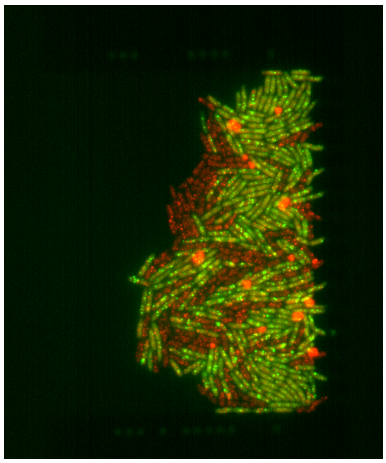
(b) The number of cells in each frame for each group of cells can be seen for the run with induced EL3354 together with EL3337.

(c) The number of cells in each frame for each group of cells can be seen for the run with induced EL3354 together with EL3336.

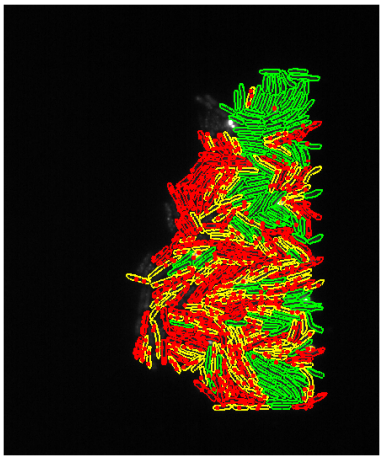
Figure 11: The following images are representing the amount of cells for each group: donor(blue), recipient (red) and transconjugant (orange), in every frame. The y-axis represents the amount of cells and the x-axis the frames. There are 241 frames in each run. All three experiments are from experiments with induced cells, so the CRISPR defense system is active. In 11a the transconjugant cells can be seen to not increase, in 11b it is possible to see a slight increase of transconjugant cells and in 11c an increase can be seen in the transconjugant cells.

3.8 Result from Image Analysis and the Tracking of Conjugation

A script called makeConjugationMovie.m was used in order to manually compare whether cells had been categorized correctly. This would be done for a couple of positions. The two fluorescent channels would be merged with each other and the image from the movie created by the script would be used to compare the spread of the conjugative plasmid and both group of cells. In Figure 12 a snapshot from one of the microfluidic runs can be seen where the two groups of bacterial cells, donors and recipients, have been mixed prior to the experiment. The image is taken after approximately one hour after the beginning of the experiment. It is possible to see the two groups of *E.coli* and it is also possible to see the spread of the RP4 plasmid, as dots in Figure 12a and as yellow cells in Figure 12b. The comparison is showing that the classifying of the cells has been accurately made since almost all cells that have red dots are either donors (red) or transconjugants (yellow) in the classifying image. Some cells are however marked as yellow when they should be donors (red) and some recipient cells are marked as transconjugants (yellow) when no obvious red dot can be seen. This can be due to the threshold in the scripts being too low or too high allowing cells to be classified into the wrong category; but the majority of the cells seem to be in the right group.



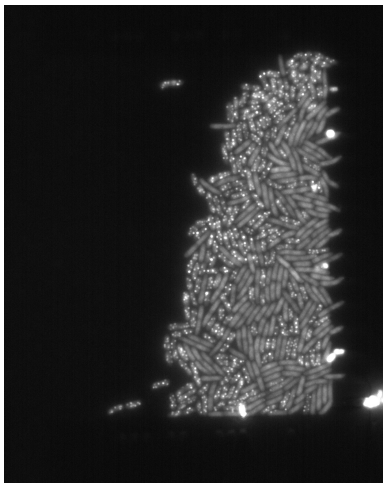
(a) The two fluorescent channels for Venus (the green marker) and mCherry (the red marker) are merged together. It is possible to see red dots in some of the green cells which indicates that the RP4 conjugative plasmid has reached the recipient cells.



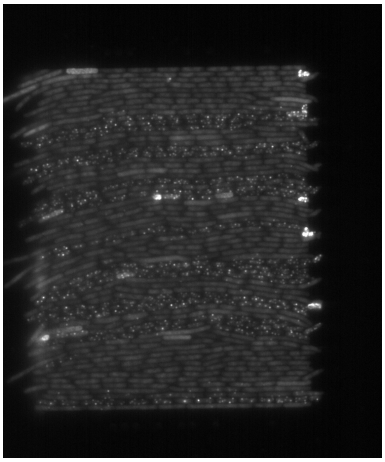
(b) Image from the makeConjugationMovie.m script which highlights recipient cells in green, transconjugant cells as yellow and donor cells as red. Red dots are also marked in the cells which indicates where the RP4 conjugative plasmid is.

Figure 12: The images is showing an example of how a comparison between the two fluorescent channels that have been merged into one image, together with an image created by the script makeConjugationMovie.m, can look. The movie is demonstrating into which class the bacterial cells should belong and where the RP4 conjugative plasmid is. It is possible to see that some recipient cells in the image to the left do contain red dots, i.e the RP4 plasmid. These cells can also be seen to be classified as transconjugant cells in the image to the right. This comparison is made manually and only to confirm that the results from the scripts are reasonable. The two groups of bacterial cells, donors and recipients, have been mixed prior to the experiment and loading.

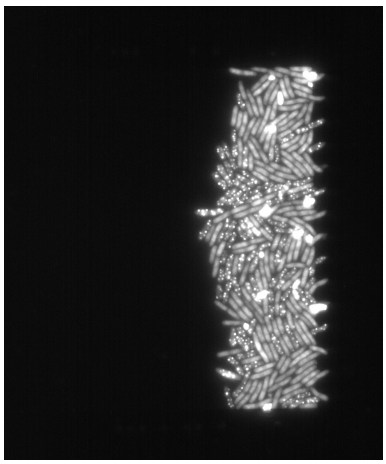
During the project, it has been noted that cells have started to die towards the end of the microfluidic experiment. The common factor is that they are induced. In Figure 13 this can be seen quite clearly. The top images have the uninduced EL3354 together with EL3336, the start and the end frame have a similar phenotype for the cells. When looking at the bottom images it is possible to see that the start frame (Figure 13c) has normal looking cells while in the last frame some cells have begun to glow (Figure 13d). The group of cells that are glowing are the recipient cells, the ones that express the CRISPR defense system. The glow that is visible from the recipient cells indicates that they are not feeling well and are probably dying. The glowing cells are also visibly longer than the non-glowing cells, which could indicate that DNA damage has happened to the bacteria (Wiktor *et al.* 2018). This was noted in all three different versions of the induced experiments (EL3354 with EL3338, EL3354 with EL3337 and EL3354 with EL3336). This was not the case when looking at the uninduced microfluidic images (Figure 13a and 13b). It seems that expressing the CRISPR defense system is also adding a stress on the cell apart from also being a target to MGEs. The glowing cells also seemed to have a slower growth rate, close to zero, which gave a very slow generation time. Therefore a cut-off was made on the growth rate. If the cell has a slower growth rate than 0.0001 [/min], it was discarded when calculating the generation time and not included in the final results. These excluded cells are also presented in tables 5 and 7 as ”Dead cells”.



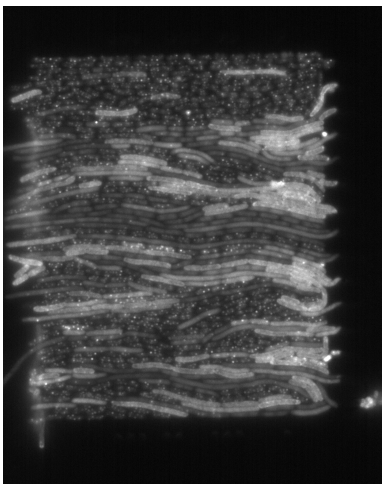
(a) The uninduced EL3354 together EL3336 can be seen in the first image of the experiment through the Red fluorescent channel.



(b) The uninduced EL3354 together with EL3336 can be seen in the last frame for the mCherry fluorescent channel.



(c) The induced EL3354 together with EL3336 can be seen in the first frame through the mCherry fluorescent channel.



(d) The induced EL3354 together with EL3336 can be seen in the last frame through the mCherry fluorescent channel. Some cells have started to glow which could indicate that they are dying.

Figure 13: A comparison between the same cells (EL3354 and EL3336) but for the induced or uninduced microfluidic experiments can be seen in the images above. Both experiments have had the donor and recipient cells mixed prior to being loaded onto the chip. The first and last frame are shown next to each other which demonstrate that the experiments with induced cells have more cells that have started to "glow". This glow together with the elongated phenotype indicates that the cell is dying. This has been a common phenomenon in all the induced experiments indicating that induction is a burden and applies stress to the cell.

4 Discussion

Two experiments for each setup (induced or uninduced) with the recipient cells and the three different donor cells were performed. In the first run, the donor cells were loaded first and then the recipient cells. This caused the donor cells to always be a the bottom of the traps, pushing the recipient cells out. Due to this, the traps were left with donor cells at the end of the run giving a low amount of recipient and transconjugant cells. In the second run of the experiments, the cells were mixed prior to loading. This led to a more even amount of recipients and donors being present in the traps. However, a problem with mixing the cells is that the cells are adjacent to each other as soon as they end up in the traps. If the loading is taking more time than anticipated

and it takes time to fill up the traps to a suitable amount of cells, then possible conjugation events could already have happened before any images have been taken. The need for speed is apparent and with training, this can be optimized. To mix the cells prior to loading is however a better approach since the connectors on the tubes do not need to be taken out of the PDMS in order to load the next cells which will not lead to PDMS dropping from the walls of the chip and clog the channels. Plus the occurrence of cells is randomized so no manual decision of when to stop loading one type of cell is needed, hence avoiding human error regarding this aspect. A problem with this approach is that the fluorescence from the cells that are emitting their light from the fluorophore might spill some of the emitted light to adjacent cells that are from another group. This could result in cells being classified into the wrong category if the threshold, that is used to classify cells, is too low or too high. When looking at the two fluorescent channels merged together and the movie made from the script makeConjugationMovie.m, and comparing these two, it is possible to see that some cells are classified into the wrong group. This could lead to the generation time being shifted towards a wrong value for the three groups of cells. However, this problem was not so great and the majority of cells seem to have been classified correctly.

4.1 The Impact of the RP4 Conjugative Plasmid

The mean division time for the donor cells and the recipient cells has been shown to differ. The recipient cells, and especially the transconjugant cells, appear to have a longer division time than the donor cells. When comparing the transconjugant cells to the donor cells, which are the newly transmitted versus the already accustomed (to the plasmid) cells, the division time was almost always greater. Other researchers have found a similar pattern in their data, where transconjugant cells that have just obtained a conjugative plasmid are dividing slower than transconjugant cells that have had the plasmid for a couple of generations (Prensky *et al.* 2021). This indicates that the generation time for newly transconjugant cells will be greater due to obtaining the conjugative plasmid. Bacterial cells that are accustomed to a certain plasmid will have a lower fitness cost than bacterial cells that have just been infected. The difference in division time was especially clear when comparing the cells when they are induced or uninduced. Throughout the experiments, the induced cells tended to have a slower division time than the uninduced cells when compared to the donor-recipient pairs (EL3354 with EL3338, EL3354 with EL3337 and EL3354 with EL3336).

It was noted that the two different approaches of loading the cells led to different amounts of bacterial cells in the different groups. The mean generation time between the two different approaches seem to vary. The one thing that can be said about the mean generation time for the first run of experiments is that there is no clear pattern. The induced EL3336 and EL3337 have a slower generation time than the uninduced version, but EL3338 has a much faster generation time than the uninduced version. The generation time for the recipient cells is also sometimes greater than for the transconjugant cells. Experiments from the first microfluidic runs would need to be rerun in order to say anything absolute from the data. A problem with the layer loading microfluidic runs is that these were the first runs that were made, therefore they contain human errors that were avoided during the second run of microfluidic experiments.

For the second approach, when mixing the cells prior to loading them onto the chip, it is possible to see some kind of pattern. All induced versions of the experiments have a slower generation time than the uninduced, which is the opposite as to what was found for the bulk growth rate

experiments. The induced EL3338 have the slowest mean generation time but the confidence interval is so great so it overlaps with induced EL3336. One conclusion from these experiments is that the transconjugant cells have the slowest generation time and especially the induced cells. This can be due to the bacteria being forced to produce Cascade and to also have obtained the RP4 conjugative plasmid. Both synthesizing the plasmid and producing Cascade will require energy from the bacteria which will cause it to slow down its division.

The data also showed that the number of transconjugant cells was lower for the version with all 18 spacer targets than for the other versions of donors together with the recipient cells. The percentage of the number of cells in the different groups had a similar pattern since the experiments with EL3338 together with EL3354 had the least amount and percentage of transconjugants. The amount of transconjugants was higher both in numbers and in percentage when looking at the EL3336 together with EL3354. The induced version had a higher amount than the uninduced which could be due to the transconjugant cells having to both produce Cascade to a random target (not the RP4 conjugative plasmid) and also having just received the plasmid and synthesizing it (table 7). The experiment with EL3337 together with EL3354 showed that the amount of transconjugant cells had decreased to almost half the amount as compared to the uninduced experiment. This pattern was not entirely the same for the first run of experiments (table 5). The difference is thought to be due to the different loading approaches which creates a biased amount of cells for the layer loading approach which affects the amount of the two groups, donors and recipients, and then ultimately the transconjugants.

A hypothesis was raised during the project which stated that since both the donor cells and recipient cells had been induced prior to the experiment, it had allowed the recipient cells to build up a set of Cascade for the targets of the RP4 plasmid. When the donor cells were adjacent to the recipient cells and conjugation events occurred, Cascade in the recipient cells would be able to recognize the conjugative plasmid as an intruder. The cell only needed to focus on targeting the plasmid and making more Cascades which should result in a lower amount of transconjugant cells in the experiments that have EL3338 together with EL3354. This is something that can be seen in the data. Both set of experiments show a low amount of transconjugant cells during experiments with EL3338. While the experiments with EL3336 have the highest amount of transconjugant cells. Since the EL3336 only had random targets for Cascade, it should not make any Cascade that is looking for the ”right” sequence in the plasmid and it should therefore be more transconjugant cells present in the data, which can also be seen when looking at the data. When looking at both the induced and uninduced experiments with EL3338 it is possible to see that both have a low amount of transconjugant cells, this could mean that some ”leakage” of Cascade expression could have happened which protected the cell against the RP4 conjugative plasmid even before the induction.

For the experiments where the donor cells had one spacer target (EL3337) for the Cascade, the division time for the transconjugant cells was slow. The long generation time is believed to have happened since Cascade had only one known target and it had been focusing on creating Cascades for the different spacers and not only the ”correct” one. The cells were therefore focusing on creating Cascades and creating the RP4 plasmid once it has entered the cell. This caused the cells to put energy into unnecessary components that were really a waste for the cell which then caused the division time to be longer. It is also possible to see that the amount of transconjugant cells is less during the experiments with induced EL3354 together with EL3337. This indicates that something has happened in the cells when they were induced, which created a protection against the RP4 conjugative plasmid as compared to when the cells were uninduced.

In the last case where there were random targets on the RP4 plasmid (EL3336) and the Cascade should not be able to recognize the plasmid as an intruder, the division time for the induced recipient cells was 135.5 minutes for the second run of experiments. This is believed to be due to the cell not knowing the RP4 to be an intruder so it is synthesizing the plasmid and creating Cascades that are looking for ”nonsense” targets. In the case for the uninduced cells, the transconjugant cells have a slower division time than the donor cells. This is believed to be due to the recipient cells gaining a new component, the RP4 plasmid, and being forced to synthesize it which requires the cell to put energy into making this plasmid. The donor cells are fairly similar in their division time and this is thought to be due to the cells already having the plasmid and ”being used to it”, this was also seen in Prensky *et al.* (2021). Therefore, the RP4 conjugative plasmid should not be a bigger burden for the cell that is already creating the component in the colony than to reallocate energy that was spent on something else before the new plasmid arrived.

4.2 The Growth Curve Tests

During the growth curve test between EL3354 and EL3203, it was noted that the induced cells appeared to have a slightly faster growth rate. The difference was 1.92 minutes between the induced and uninduced EL3354 and 1.44 minutes between the induced and uninduced EL3203. The difference is not big but it can play an important role for the cell and determine whether it can compete with other cells regarding food and space. It is also interesting that the induced cells had a faster generation time since these cells were producing the CRISPR defense system. In theory, these cells should be slower. The data obtained in this project is showing the opposite of what Vink *et al.* found (2020). They saw that the induced strains were slower than the uninduced cells that had similar components as the recipient cells that are used in this project. It is also unclear why the uninduced cells were slightly slower than the induced cells used in this project. When growth curve tests were made for the versions of the recipient cells that did not have all the components as the cells used during the microfluidic experiments it was noted that both pre-recipient cells had a faster generation time than the recipient cells used in the project. The pre-recipient cells have the same genotype as the recipient cells, the only difference is that they are lacking the plasmid that helps to induce the expression of Cascade and Cas3. The pre-recipient cells to EL3354 (strain EL3351) had the same mean generation time at 27.5 minutes but the difference between the recipient cells were 5.21 minutes slower for the induced cells and 7.13 minutes slower for the uninduced. The uninduced recipient cells (EL3354) were 1.92 minutes slower than the induced cells. This indicates that the components that have been added to the recipient strain affect the growth rate and with that the generation time of the cells. The same pattern was found for the pre-recipient cells for the EL3203 (strain EL3202) also indicating that the new components that have been added to the strain are affecting the generation time, see Appendix C tables 9 and 10.

The growth curve test between the donors EL3336, EL3337 and EL3338 showed that they were all around 29 minutes for each generation. The one outlier, induced EL3338, is believed to be due to the flask and its earlier content (Appendix C table 11). The flask was the only component that was different from the other vials. All the flasks with bacterial cultures were treated the same, so the only element that could cause the difference is the flask itself.

When comparing the division time between cells in a flask and cells on a chip it can be noted

that the cells on the chip are dividing slower. This could be due to the space being very limiting which makes it harder for the cells to divide since they need to compete about the small space. The temperature is also different, as the bulk experiment had 37°C and the temperature in the chip was at 30°C. Another factor is that the cells are not getting as much oxygen as the cells in the flask, which can also be a factor in the cells dividing slower. The cells in the chip are not feeling well, which is apparent when looking at images 13c and 13d. In image 13d it is possible to see that cells have started to glow and elongate at the end of the experiment, indicating that they are dying. One possible cause of this can be that CRISPR is targeting itself, since this is not happening when the cells are uninduced and this ”glow” occurred with all three different versions. This has been the case for other researchers that have noticed that self-targeting spacers have been a part of the CRISPR array (Devi *et al.* 2022). It therefore seems that prokaryotes could also suffer from autoimmunity. Another interesting phenomenon was that induced recipient cells were dividing faster than the uninduced recipient cells in the bulk experiments. While the induced recipient cells were dividing significantly slower than the uninduced recipient cells when they were in the PDMS chip. No explanation has yet been found as to why this was happening.

Nevertheless, more research is needed and reproduction of the experiments in order to be confident with what has been seen in this project.

4.3 Is It Possible to Use CRISPR in Order to Fight Antibiotic Resistance?

The hopeful end-goal for this project could be to use what has been learned in a pharmaceutical setup in order to fight antibiotic resistance. The worldwide health crisis that exists due to the antibiotic resistance in bacteria is causing an earlier controlled infection to become possibly deadly (Ventola 2015). By overusing antibiotics in both humans and animals, resistance has beginning to form in bacteria and it is particularly clear in specific regions around the world. A possible use of CRISPR as a tool against antibiotic resistance is to use the gene-editing scissor in a commensal bacteria. For example, one of the bacteria used in yogurt could possibly be used here. A probiotic with a CRISPR array that recognizes the sequence from an antibiotic resistant gene could be added in all of the spacers which would ensure that the antibiotic gene inside the bacteria will be targeted.

A need in this setup would be to control the conjugative plasmid that is transferring the CRISPR system to a bacteria that contains an antibiotic resistant gene. By controlling the plasmid and with this also the transfer of the CRISPR system, the approach would be safer and the possible spread of an unwanted plasmid could be hindered. For example, one way to control the conjugative plasmid could be to use bisphosphonate, which would then inhibit the relaxase activity (Lujan *et al.* 2007). The relaxase is the component that nicks the plasmid that is going to be transferred to a new cell, so if the plasmid can not be nicked and turned into linear DNA then no transfer can happen. If a probiotic could be manufactured that had the CRISPR system, targeting a specific antibiotic resistant gene, which could be transferred with the help of a conjugative plasmid and to also control the plasmids transfer activity, then a possible solution could be reached for antibiotic resistance worldwide. Lujan *et al.* showed that they could control the relaxase activity in F plasmids and by that stopping DNA transfer. Their experiments were however done *in vitro*. Another research group has been proposing to use conjugative plasmid in order to fight antibiotic resistant genes in wastewater treatment plants (Li *et al.* 2022). They propose using the conjugating bacteria in one of the purifying steps. One problem with this approach is

that the conjugative plasmid will be left in the microorganisms even if the antibiotic resistant genes have been destroyed. Therefore studies of how the conjugative plasmid is behaving in a larger eco-system are needed and studies that demonstrate how the plasmid can be controlled. Nevertheless, several research groups are looking into the possibilities to use CRISPR in order to fight antibiotic resistant genes.

The answer to the question whether CRISPR can be used to fight antibiotic resistance is 'maybe'. Since CRISPR is the natural defense against invading MGEs and it can locate specific sequences in a cell, it could be possible to use. The problem seems to lie in how to control the transfer of CRISPR, in what organism the CRISPR system should be expressed and how to control unwanted spread of the conjugative plasmid. Therefore, the conclusion is maybe, but leaning towards yes. If the logistics and technical issues regarding the distribution of CRISPR in a safe manner can be achieved, then this could be a possible approach.

5 Conclusion

The conclusion from this master thesis is that it is possible to stop the spread of a conjugative plasmid and that the defense mechanism, CRISPR, in prokaryotic organisms is effective. The more spacer targets that are available will result in a better defense and fewer transconjugant cells will be made. It is also apparent that the CRISPR defense mechanism can be an energy burden that hinders the cell from dividing. CRISPR can also be harmful to the cell if it targets itself. More research will be needed regarding this subject but this master thesis has helped to lay the puzzle.

6 Acknowledgment

I want to thank my supervisor, Daniel Jones, for letting me be part of this project and giving me the opportunity to do my Master Thesis project at Jones lab at Uppsala Univeristy. I have learned so much and grown as an individual and I feel really honored that you believed in my ability to contribute to this project. I would also like to thank my subject reviewer, Johan Elf, for helping my to take import steps and decisions during the project and to also having valuable discussions that helped me to take my project further. I would like to thank Jimmy Larsson for teaching me (almost) everything that I know about microfluidics, PDMS chips and fluorescent microscopy and for always being present to answer any questions that I had regarding anything in the lab or microfluidics. I would like to thank Konrad Gras for lending me your knowledge and time. I would like to thank Spartak Zikrin for helping me with the ImAnalysis pipeline and to also help me understand how to build my scripts and my folders in Matlab in the best possible way. I would like to thank Jakub Wiktor for helping both me and my supervisor to improve the segmentation of the cells with a new AI network which helped me to get my final results that are presented in this report.

I would also like to thank my examinator, Johan Åqvist, my course administrator Lena Henriksson, and my opponent, Emelia Karlsson, for providing me with feedback and help during my master thesis.

Lastly but not least I would like to thank the Elflab, Johansson and Jones lab and all the people in them for being the wonderful individuals that they are and for letting me being a part of your lab and welcoming me during my master thesis project.

References

Akinsheye I, Alsultan A, Solovieff N, Ngo D, Baldwin CT, Sebastiani P, Chui DH, Steinberg MH. 2011. Fetal hemoglobin in sickle cell anemia. Blood, The Journal of the American Society of Hematology 118: 19–27.

Arslan Z, Stratmann T, Wurm R, Wagner R, Schnetz K, Pul Ü. 2013. RcsB-BglJ-mediated activation of cascade operon does not induce the maturation of CRISPR RNAs in *E. coli* K12. RNA biology 10: 708–715.

Badrinarayanan A, Le TB, Laub MT. 2015. Bacterial chromosome organization and segregation. Annual review of cell and developmental biology 31: 171–199.

Barrangou R. 2015. The roles of CRISPR–Cas systems in adaptive immunity and beyond. Current Opinion in Immunology 32: 36–41.

Baylis F, McLeod M. 2017. First-in-human phase 1 CRISPR gene editing cancer trials: are we ready? Current gene therapy 17: 309–319.

Bragheri F, Martínez Vázquez R, Osellame R. 2020. Chapter 12.3 - Microfluidics. Baldacchini T, editor, Three-Dimensional Microfabrication Using Two-Photon Polymerization (Second Edition), William Andrew Publishing, Micro and Nano Technologies, 493–526.

Devi V, Harjai K, Chhibber S. 2022. Self-targeting spacers in CRISPR-array: Accidental occurrence or evolutionarily conserved phenomenon. Journal of Basic Microbiology 62: 4–12.

Getino M, de la Cruz F. 2018. Natural and artificial strategies to control the conjugative transmission of plasmids. Microbiology spectrum 6: 6–1.

Greely HT. 2019. CRISPR'd babies: human germline genome editing in the 'He Jiankui affair'. Journal of Law and the Biosciences 6: 111–183.

Hao JJ, Yarmolinsky M. 2002. Effects of the P1 plasmid centromere on expression of P1 partition genes. Journal of bacteriology 184: 4857–4867.

Jones DL, Leroy P, Unoson C, Fange D, Ćurić V, Lawson MJ, Elf J. 2017. Kinetics of dCas9 target search in *Escherichia coli*. Science 357: 1420–1424.

Kim M, Park J, Kang M, Yang J, Park W. 2021. Gain and loss of antibiotic resistant genes in multidrug resistant bacteria: One health perspective. Journal of Microbiology 1–11.

Lambert T. 2022a. mCherry at FPbase.

Lambert T. 2022b. SCFP3A at FPbase.

Lambert T. 2022c. Venus at FPbase.

Li X, Bao N, Yan Z, Yuan XZ, Wang SG, Xia PF. 2022. Tailoring CRISPR-Cas immunity for the degradation of antibiotic resistance genes. bioRxiv .

Liu G, Lin Q, Jin S, Gao C. 2021. The CRISPR-Cas toolbox and gene editing technologies. Molecular Cell .

Lujan SA, Guogas LM, Ragonese H, Matson SW, Redinbo MR. 2007. Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase. Proceedings of the National Academy of Sciences 104: 12282–12287.

Maeder ML, Stefanidakis M, Wilson CJ, Baral R, Barrera LA, Bounoutas GS, Bumcrot D, Chao H, Ciulla DM, DaSilva JA, *et al.* 2019. Development of a gene-editing approach to restore vision loss in leber congenital amaurosis type 10. Nature medicine 25: 229–233.

Marraffini LA, Sontheimer EJ. 2008. CRISPR interference limits horizontal gene transfer in *Staphylococci* by targeting DNA. science 322: 1843–1845.

Nikon. 2022. ECLIPSE Ti2 Series | Specifications.

Nobel Prize. 2020. The Nobel Prize in Chemistry 2020.

Nordström K, Austin SJ. 1989. Mechanisms that contribute to the stable segregation of plasmids. Annual review of genetics 23: 37–69.

Pelgrift RY, Friedman AJ. 2013. Nanotechnology as a therapeutic tool to combat microbial resistance. Advanced drug delivery reviews 65: 1803–1815.

Prensky H, Gomez-Simmonds A, Uhlemann AC, Lopatkin AJ. 2021. Conjugation dynamics depend on both the plasmid acquisition cost and the fitness cost. Molecular systems biology 17: e9913.

Raleigh EA, Low KB. 2013. Conjugation. Maloy S, Hughes K, editors, Brenner's Encyclopedia of Genetics (Second Edition), Academic Press, San Diego, 144–151.

San Millan A, MacLean RC. 2017. Fitness costs of plasmids: a limit to plasmid transmission. Microbiology spectrum 5: 5–5.

Savvides SN, Raghunathan S, Fütterer K, Kozlov AG, Lohman TM, Waksman G. 2004. The C-terminal domain of full-length *E. coli* SSB is disordered even when bound to DNA. Protein Science 13: 1942–1947.

Shen Z, Tang CM, Liu GY. 2022. Towards a better understanding of antimicrobial resistance dissemination: what can be learnt from studying model conjugative plasmids? Military Medical Research 9: 1–11.

Squires TM, Quake SR. 2005. Microfluidics: Fluid physics at the nanoliter scale. Reviews of modern physics 77: 977.

Synthego. 2022. What is the PAM Sequence in CRISPR Experiments, and Why is it Important?

Uddin F, Rudin CM, Sen T. 2020. CRISPR gene therapy: applications, limitations, and implications for the future. Frontiers in Oncology 10: 1387.

Van Boeckel TP, Gandra S, Ashok A, Caudron Q, Grenfell BT, Levin SA, Laxminarayan R. 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data. The Lancet infectious diseases 14: 742–750.

Ventola CL. 2015. The antibiotic resistance crisis: part 1: causes and threats. Pharmacy and therapeutics 40: 277.

Vink JN, Martens KJ, Vlot M, McKenzie RE, Almendros C, Bonilla BE, Brocken DJ, Hohlbein J, Brouns SJ. 2020. Direct visualization of native CRISPR target search in live bacteria reveals cascade DNA surveillance mechanism. Molecular Cell 77: 39–50.

Vrancianu CO, Popa LI, Bleotu C, Chifiriuc MC. 2020. Targeting plasmids to limit acquisition and transmission of antimicrobial resistance. Frontiers in microbiology 11: 761.

Wallden M, Fange D, Lundius EG, Baltekin Ö, Elf J. 2016. The synchronization of replication and division cycles in individual e. coli cells. Cell 166: 729–739.

Weibel DB, DiLuzio WR, Whitesides GM. 2007. Microfabrication meets microbiology. Nature Reviews Microbiology 5: 209–218.

Whitesides GM. 2006. The origins and the future of microfluidics. nature 442: 368–373.

WHO. 2021. Antimicrobial resistance.

Wiktor J, Van Der Does M, Büller L, Sherratt DJ, Dekker C. 2018. Direct observation of end resection by RecBCD during double-stranded DNA break repair *in vivo*. Nucleic acids research 46: 1821–1833.

Xue C, Sashital DG. 2019. Mechanisms of type IE and IF CRISPR-Cas systems in *Enterobacteriaceae*. EcoSal Plus 8.

Zhang J, Hoshino K. 2019. Chapter 2-fundamentals of nano/microfabrication and effect of scaling. Molecular Sensors and Nanodevices 43–101.

A Microscope settings and technical data

A.1 Microscope technical specification

A.1.1 The microscope

Main body FPGA (Field Programmable Gate Array):0130

Transmitted light comes from a Nikon LED and uses LWD (Long Working Distance) condenser lens.

Two objectives were used with corresponding phase plates: Plan Fluor, 20X, NA 0.5, Ph1, MRH10201 Plan Apo Lambda, 100X, NA 1.45, Ph3, MRD31905

The light source used was the Lumencore-Spectra III which is attached via a TI2-LA-BF and a TI2-LA-FLL

The Camera is a Teledyne Photometrics

The incubator for the microscope that ensures an even and wanted temperature is an Okolab-H201-Enclosure hood and a H201-T-UNIT-BL temperature control unit.

A.1.2 Filter cubes

Thorlabs 520E, Dichroic mirror Di02-R514 (Semrock). The excitation wavelength is 510 nm and the emission wavelength is 514 nm.

Nikon 593A, Dichroic mirror FF593-Di03 (Semrock). The excitation was at wavelength 562 nm and the emission was at wavelength 624 nm.

Nikon Brightline Di02-R442 (Semrock). The excitation is at wavelength 433 nm and emission at wavelength 474 nm.

B Calculations

The intensity of the light that excites the fluorescent tag was measured by collecting the out-coming light at a specific wavelength with a sensor. The out-coming light was set at the intensity of the corresponding light used in the experiment. The value that was measured was then noted as the watt that is reaching the sample. The area that is used in the calculations was measured by letting the light go through the most narrow setting possible. This light was then imaged with the camera and a small square could be seen through the program ImageJ. The area for this light, measured in pixels, was noted to be 6624 pixels with the 20x magnification. Now the watt for the out-coming light and the area of the pixels had been measured.

In order to calculate the area in cm^2 the size of the pixel was needed, which was 6.5 μm.

$$Pixel\ Area = (6.5 * 10^{-6})^2 = 4.225 * 10^{-11} m^2$$

$$Area = (Amount\ of\ Pixels) * \frac{Pixel\ Area}{Magnification} = 6624 \frac{4.225 * 10^{-11}}{20} = 1.39932 * 10^{-8} m^2 =$$

$$= 1.39932 * 10^{-4} cm^2$$

In order to calculate the power density for each light coming from the light source, the out-coming energy was measured, see table 8. A light sensor was held directly at the same position as the sample would be and all the outcoming light was collected.

Table 8: The intensities from the out-coming light measured in Watt can be seen in the table below.

Light source name	Wavelength [nm]	Watt [mW]	Power Intensity W/cm^2
Blue	442	5.75	41.09
Teal	514	1.44	10.27
Red	594	2.97	21.22

The power density for the Blue light at wavelength 442 nm was calculated according to:

$$Power\ Density = \frac{W}{cm^2} = \frac{5.75 * 10^{-3}}{1.39932 * 10^{-4}} = 41.09\ W/cm^2$$

The power density for the Teal light at wavelength 514 nm was calculated according to:

$$Power\ Density = \frac{W}{cm^2} = \frac{1.437 * 10^{-3}}{1.39932 * 10^{-4}} = 10.27\ W/cm^2$$

The power density for the Red light at wavelength 594 nm was calculated according to:

$$Power\ Density = \frac{W}{cm^2} = \frac{2.97 * 10^{-3}}{1.39932 * 10^{-4}} = 21.22\ W/cm^2$$

C Generation Time

During the microfluidic experiments, it was noted that the recipient cells appeared to be growing at a different rate. Therefore a growth curve experiment was performed.

The generation time will be calculated with the help of μ, which is a constant that is specific to the organism used. O/N cultures were made of the strains that were included in the growth curve test (EL3203, EL3354, EL3202, EL3351, EL3336, EL3337 and EL3338). These were diluted 1:2000 the next day and incubated at 37°C for 2 hours before measurements every 30 minutes were made. The result can be seen in tables 9, 10 and 11.

Here is an example of how the generation time was calculated.

$$\mu = \frac{ln(2)}{Generation\ time}$$

$$Generation\ time = \frac{ln(2)}{\mu}$$

$$= \frac{ln(2)}{1.32} * 60 = 31.5\ minutes$$

The generation time and the μ value for the recipient cells can be found in the table below (table 9). Two growth curve experiments were made for every version of the strain and its induced or uninduced state.

Table 9: The cells described in the table below are the recipient cells used in the microfluidic experiments, the EL3354 and EL3203. The growth rates and generation time are calculated with the formula in the equation above. μ *is the specific growth rate that can be obtained by plotting LN(OD600) vs the time. Induced cells are marked with + and uninduced cells with -. The confidence interval, shown in the brackets, is based on the standard error of the mean (±SEM).*

Strain +/-	μ	Generation time [min]	μ	Generation time [min]	Mean [min]
EL3354 -	1.23	33.8	1.17	35.5	34.6 (±1.2)
EL3354 +	1.32	31.5	1.23	33.9	32.7 (±1.7)
EL3203 -	1.25	33.3	1.30	34.5	33.7 (±0.5)
EL3203 +	1.33	31.3	1.22	32.0	31.7 (±0.5)

Table 10: The cells in this table are the pre-recipient cells, the recipient cells before they have all the components that are used in the microfluidic experiments, the EL3351 and EL3202. The growth rates and generation time are calculated with the formula in the equation above. μ is the specific growth rate that can be obtained by plotting LN(OD600) vs the time. Induced cells are marked with + and uninduced cells with -. The confidence interval shown in the brackets is based on the standard error of the mean (±SEM).

Strain +/-	μ	Generation time [min]	μ	Generation time [min]	Mean [min]
EL3351 -	1.56	26.6	1.47	28.4	27.5 (±1.3)
EL3351 +	1.58	26.4	1.46	28.5	27.5 (±1.5)
EL3202 -	1.37	30.3	1.47	28.3	29.3 (±1.4)
EL3202 +	1.33	31.0	1.43	29.1	30.1 (±1.3)

The generation time and μ values for the donor cells can be found in the table below (table 11). Two growth curve experiments were made for every version of the strain and its induced or uninduced state.

*Table 11: The generation time for the donor cells can be seen in this table. The growth rates and generation time are calculated with the formula in the equation above. μ is the specific growth rate that can be obtained by plotting LN(OD600) vs the time. Induced cells are marked with + and uninduced cells with -. The * is marking the value that has been excluded from the mean value due to uncertainty. The value for EL3338+ in the second experiment is thought to be due to the flasks' earlier content, which was unfavorable for the bacteria giving rise to a slower generation time. The confidence interval shown in the brackets is based on the standard error of the mean (±SEM).*

Strain +/-	μ	Generation time [min]	μ	Generation time [min]	Mean [min]
EL3336 -	1.54	27.0	1.37	30.3	28.6 (±2.3)
EL3336 +	1.47	28.3	1.33	31.3	29.8 (±2.1)
EL3337 -	1.45	28.7	1.41	29.6	29.2 (±0.6)
EL3337 +	1.45	28.7	1.42	29.4	29.1 (±0.5)
EL3338 -	1.48	28.2	1.40	29.7	28.9 (±1.0)
EL3338 +	1.47	28.3	0.97	43.0*	28.3 (± N/A)

The median growth rate for the layer-loading approach of the three different groups can be seen in table 12. This value is important since it describes where the majority of the growth rates are.

Table 12: The median for the different strains and whether they are induced or uninduced can be seen in the following table. The median was calculated with the help of the "median" function in MATLAB. Only the cells that passed the criteria were included.

Cell type	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Induction	-	+	-	+	-	+
Donor	45.3	42.5	39.3	44.4	43.2	43.1
Recipient	49.9	48.1	42.2	49.7	44.6	46.4
Transconjugant	49.4	45.4	42.7	51.4	44.8	44.8

The median for the mixing prior to loading approach for the different groups of cells and for all microfluidic experiments performed during the second run of experiments, or when the cells were mixed prior to loading, which can be seen in table 13.

Table 13: The median for the different strains and whether they are induced or uninduced can be seen in the following table. The median was calculated with the help of the "median" function in MATLAB. Only the cells that passed the criteria were included.

Cell type	EL3338	EL3338	EL3337	EL3337	EL3336	EL3336
Induction	-	+	-	+	-	+
Donor	45	44.3	46.2	43.7	44.9	44.5
Recipient	45.6	58.2	47.3	48	45.9	49.9
Transconjugant	46.9	48.4	47.9	46.7	46.8	51.2

D The script: analyzeRunConjugationSSB.m

```
%Load in expInfoObj file with an absolute path and define variables
 that
%are used in the script
expInfoFile= '/crex/proj/uppstore2018129/elflab/Projects/
CRISPR_conjugation/Analysis2/EXP-22-BY4448/therun/prod/
expInfoObj.mat';
load(expInfoFile);
threshold= '/crex/proj/uppstore2018129/elflab/Projects/
CRISPR_conjugation/Analysis2/EXP-22-BY4448/therun/prod/
SSBThresholdValues.txt'; % note from DJ: this needs to be higher,
 maybe 400 or so
load(threshold);
phaseChanName='phase';

finalDonorYFPFluo=[];
finalRecipientYFPFluo=[];
finalTransconjugantYFPFluo=[];
growthRateDonor=[];
growthRateRecipient=[];
growthRateTransconjugant=[];
positionList=expInfoObj.positions;



phaseIdx =
 expInfoObj.getIndices(positionList{1},phaseChanName); %making
numberDonorsPerFrame=zeros(length(phaseIdx),1); %count the amount of
 donors for each frame
numberRecipientsPerFrame=zeros(length(phaseIdx),1);
numberTransconjugantsPerFrame=zeros(length(phaseIdx),1);
```

Loop through positions and perform classifying of cells and cell fluorescence

```
for a=1:length(positionList)

    computeCellFluorescence(expInfoObj,
 positionList{a}, 'fluor_YFP_venus');
    classifyCellsConjugationStatus(expInfoObj,
 positionList{a}, 'fluor_YFP_venus', 'fluor_594_cherry', threshold);
    inputFile=fullfile(expInfoObj.outputFolderPath,
 positionList{a}, 'trackedCells.mat');
    load(inputFile);

    nCells=length(mCells());
    currMeanVenus=zeros(nCells,1);
    ndonor= 0;
    nrecip=0;
    ntrans=0;
```

```
  %Count how many cells there are from each group
   for i=1:nCells
       if logical([mCells(i).isBadCell]) == 0
           if mCells(i,1).isDonor
               ndonor=ndonor +1;
           elseif mCells(i,1).isRecipient
               nrecip= nrecip +1;
           elseif mCells(i,1).isTransconjugant
               ntrans=ntrans +1;
           end
       end
   end
   %headLine= categorical({'Donors', 'Recipient', 'Transconjugant'});
   %headLine=reordercats(headLine,{'Donors', 'Recipient',
'Transconjugant'});
   %b=[ndonor, nrecip, ntrans];

   %figure, bar(headLine, b);

   donorInd=1;
   donorIndex=zeros(ndonor, 1, 'uint32');
   donorMeanFluo=zeros(ndonor,1);
   recipInd=1;
   recipIndex= zeros(nrecip,1, 'uint32');
   recipientMeanFluo=zeros(nrecip,1);
   transInd=1;
   transIndex=zeros(ntrans,1, 'uint32');
   transconjugantMeanFluo=zeros(ntrans,1);

   for j=1:nCells
       if (logical([mCells(j).isBadCell]) == 0) &&
(~isempty(mCells(j).descendants) || ~isempty(mCells(j).parent))
       currMeanVenus(j)=nanmean(mCells(j).fluoIntensities(2,:));
       if mCells(j).isDonor
           donorMeanFluo(donorInd)=currMeanVenus(j);
           donorIndex(donorInd)=j;
           donorInd=donorInd+1;
       elseif mCells(j).isRecipient
           recipientMeanFluo(recipInd)=currMeanVenus(j);
           recipIndex(recipInd)=j;
           recipInd=recipInd +1;
       else
           transconjugantMeanFluo(transInd)=currMeanVenus(j);
           transIndex(transInd)=j;
           transInd=transInd+1;
       end
       end
   end

   for k=1:length(phaseIdx)
       for l=1:nCells
           if (phaseIdx(k)>= mCells(l).birthFrame) &&
(phaseIdx(k)<= (mCells(l).birthFrame + mCells(l).lifeTime)) &&
(~isempty(mCells(j).descendants) || ~isempty(mCells(j).parent))
```

```
                if mCells(l).isDonor
                    numberDonorsPerFrame(k)=numberDonorsPerFrame(k)
+1;
                elseif mCells(l).isRecipient

numberRecipientsPerFrame(k)=numberRecipientsPerFrame(k) +1;
                else

numberTransconjugantsPerFrame(k)=numberTransconjugantsPerFrame(k) +1;
                end
            end
        end
    end


    %disp(positionList{a})

Starting parallel pool (parpool) using the 'local' profile ...
Connected to the parallel pool (number of workers: 4).

    [cellGrowth, cellInd, cellStd]= getGrowthRates(mCells);

    %Comparing index from array with venus fluorescent values and the
    %indexes obtained from getGrowthRates. Save the growthrates from
the
    %for each cell group
    [~,donorGrowthIdx,donorFluoIdx]=intersect(cellInd,donorIndex);
    donorGrowth=cellGrowth(donorGrowthIdx);
    donorFluoYFP=donorMeanFluo(donorFluoIdx);

    [~,
recipientGrowthIdx,recipientFluoIdx]=intersect(cellInd,recipIndex);
    recipientGrowth=cellGrowth(recipientGrowthIdx);
    recipFluoYFP=recipientMeanFluo(recipientFluoIdx);


[~,transconjugantGrowthIdx,transconjugantFluoIdx]=intersect(cellInd,
transIndex);
    transconjugantGrowth=cellGrowth(transconjugantGrowthIdx);
    transFluoYFP=transconjugantMeanFluo(transconjugantFluoIdx);

    %concatenating all fluorescent values from the array and the
growthrate
    %for each cell group
    finalDonorYFPFluo=cat(1,finalDonorYFPFluo, donorFluoYFP);
    growthRateDonor= cat(2, growthRateDonor, donorGrowth);
    finalRecipientYFPFluo=cat(1,finalRecipientYFPFluo, recipFluoYFP);
    growthRateRecipient= cat(2, growthRateRecipient, recipientGrowth);
    finalTransconjugantYFPFluo=cat(1,finalTransconjugantYFPFluo,
transFluoYFP);
    growthRateTransconjugant= cat(2, growthRateTransconjugant,
transconjugantGrowth);

end
```

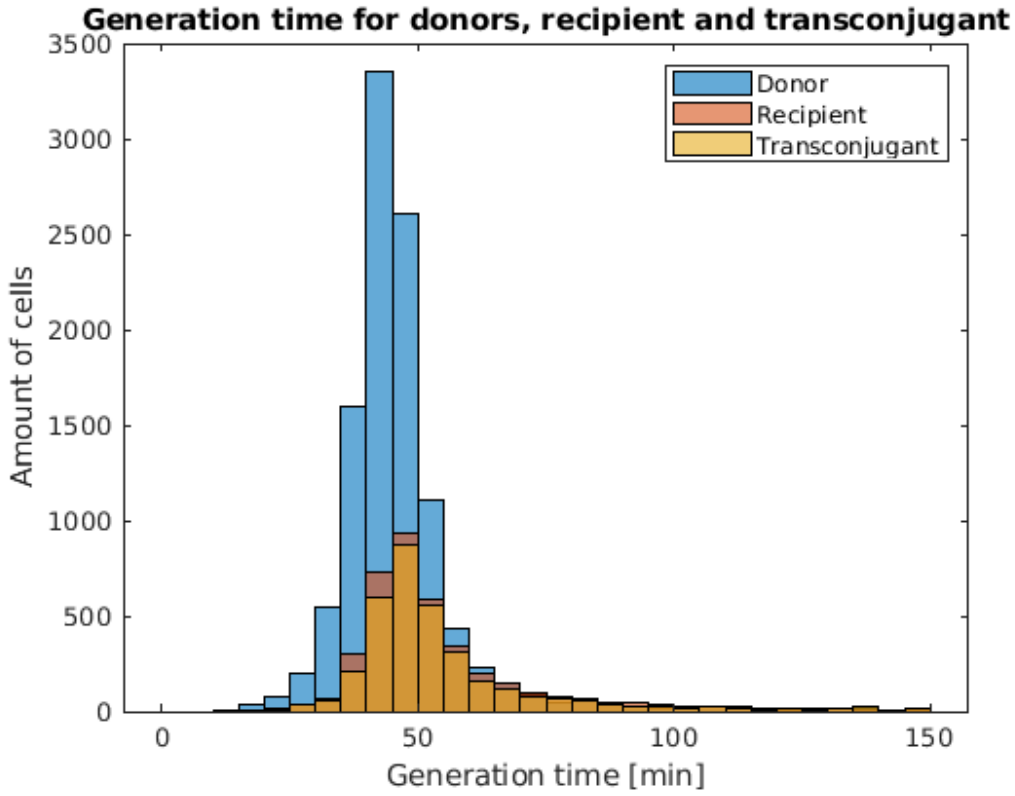
Collecting the data

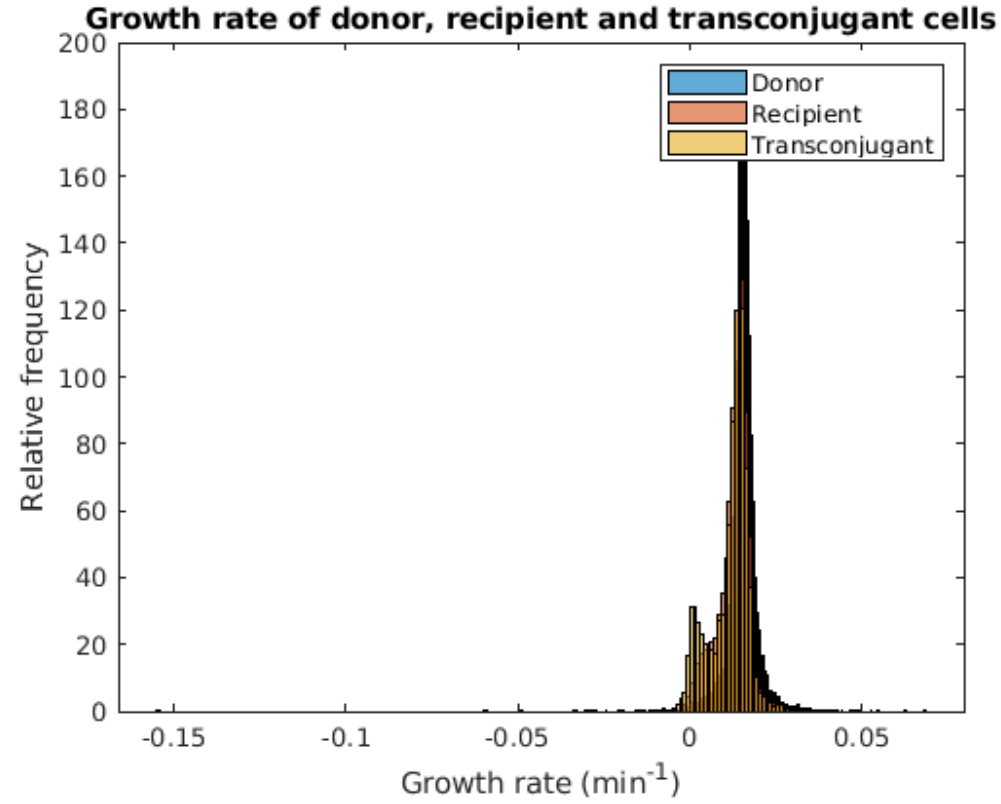
```
%Calculating the generation time for each cell and then taking the
 mean of
%them
edges=0:5:150;

%changed to cut at 0.0001 growth rate to avoid dying cells but still
%including as many cells as possible
genTimeMiddleDonor=growthRateDonor(1,:)>0.0001;
genTimePosDonor=growthRateDonor(:,genTimeMiddleDonor);

genTimeMiddleRecipient=growthRateRecipient(1,:)>0.0001;
genTimePosRecipient=growthRateRecipient(:,genTimeMiddleRecipient);

genTimeMiddleTransconjugant=growthRateTransconjugant(1,:)>0.0001;
genTimePosTransconjugant=growthRateTransconjugant(:,genTimeMiddleTransconjugant);

%calculating the generation time for each cell found from the data
genTimeDonor=(log(2)./genTimePosDonor);
genTimeRecipient=(log(2)./genTimePosRecipient);
genTimeTransconjugant=(log(2)./genTimePosTransconjugant);

%Calcuating the mean generation time for each cell group
meanGenTimeDonor=mean(genTimeDonor);
meanGenTimeRecipient=mean(genTimeRecipient);
meanGenTimeTransconjugant=mean(genTimeTransconjugant);

%Calculate the confidence interval
SEMDon = std(genTimeDonor)./sqrt(length(genTimeDonor));
  % Standard Error
tsDon = tinv([0.025  0.975],length(genTimeDonor)-1);      % T-Score
 for 95% of the data
CIDon = mean(genTimeDonor) + tsDon*SEMDon; %get value, lower and
 higher from the mean
CIstrDon='Confidence intervall for Donor growth [lower higher]: ';
strMeanGrowth=' The mean growth rate is: ';
%write the result together with the string
outCIDon=([CIstrDon,num2str(CIDon), newline,
 strMeanGrowth, num2str(mean(genTimeDonor)), newline,
 num2str(median(genTimeDonor))]);

%Confidence interval for recipient cells
SEMRec = std(genTimeRecipient)./sqrt(length(genTimeRecipient));
        % Standard Error
tsRec = tinv([0.025  0.975],length(genTimeRecipient)-1);      % T-
Score
CIRec = mean(genTimeRecipient) + tsRec*SEMRec; %get value, lower and
 higher from the mean
CIstrRec='Confidence intervall for Recipient growth [lower higher]: ';
outCIRec=([CIstrRec,num2str(CIRec), newline,
 strMeanGrowth, num2str(mean(genTimeRecipient)), newline,
 num2str(median(genTimeRecipient))]);
```

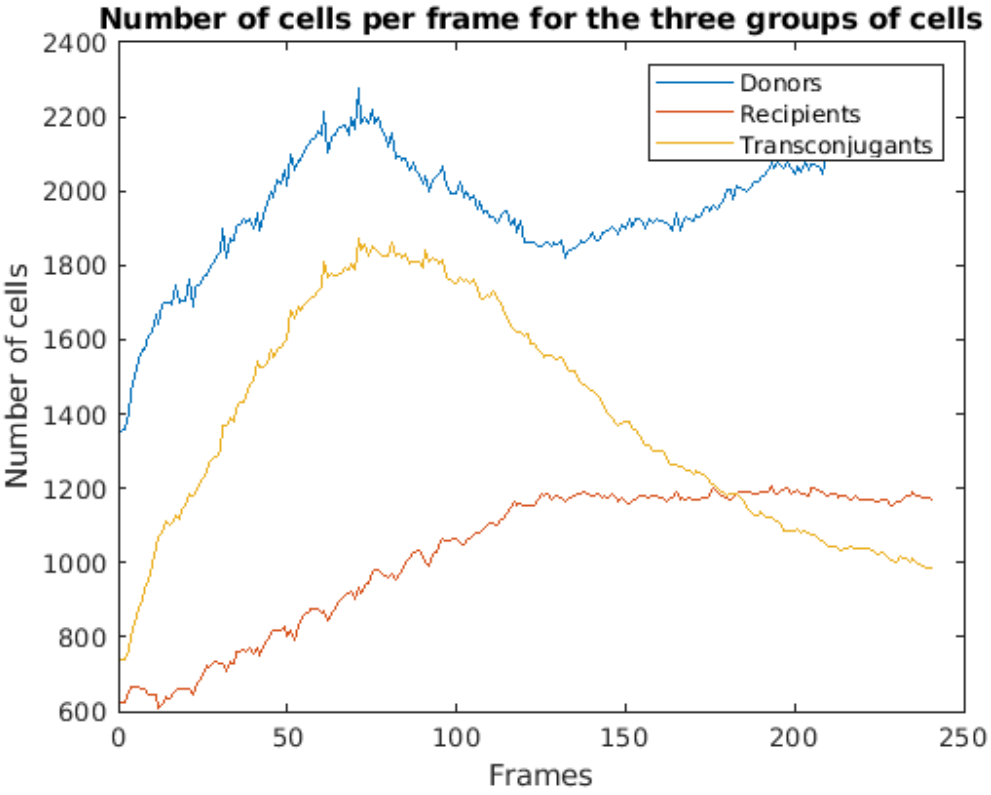
```
%confidence interval for transconjugant cells
SEMTra = std(genTimeTransconjugant)./
sqrt(length(genTimeTransconjugant));               % Standard Error
tsTra = tinv([0.025  0.975],length(genTimeTransconjugant)-1);      %
 T-Score
CITra = mean(genTimeTransconjugant) + tsTra*SEMTra; %get value, lower
 and higher from the mean
CIstrTra='Confidence intervall for Transconjugant growth [lower
 higher]: ';
outCITra=([CIstrTra,num2str(CITra), newline,
 strMeanGrowth,num2str(mean(genTimeTransconjugant)), newline,
 num2str(median(genTimeTransconjugant))]);

%creating a histogram for the generation times and saving them
histogramOfGenerationTime=figure;
histogram(genTimeDonor, edges);
hold on
histogram(genTimeRecipient,edges);
histogram(genTimeTransconjugant, edges);
legend('Donor', 'Recipient', 'Transconjugant');
title('Generation time for donors, recipient and transconjugant');
xlabel('Generation time [min]');
ylabel('Amount of cells');
outputFileNameGenTime =
 fullfile(expInfoObj.outputFolderPath, 'generation-
time_donor_recip_trans.fig');
savefig(outputFileNameGenTime);
outputFileNamePNGGenTime =
 fullfile(expInfoObj.outputFolderPath, 'generation-
time_donor_recip_trans.png');
exportgraphics(histogramOfGenerationTime,outputFileNamePNGGenTime)
hold off

%Calculate the amount of cells for each group and also writing out how
 many
%cells that are excluded from the data
amountDon=length(genTimeDonor);
amountLeftDon=length(growthRateDonor)-length(genTimeDonor);
amountRec=length(genTimeRecipient);
amountLeftRec=length(growthRateRecipient)-length(genTimeRecipient);
amountTra=length(genTimeTransconjugant);
amountLeftTra=length(growthRateTransconjugant) -
length(genTimeTransconjugant);
strDon='The mean division time for the donor cells are: ';
strRec='The mean division time for the recipient cells are: ';
strTra='The mean division time for the transconjugant cells are: ';
strEnd=' minutes.';
strAmountDon=' The amount of cells for the donors are:';
strAmountRec=' The amount of cells for the recipients are:';
strAmountTra=' The amount of cells for the transconjugants are:';
strLeft='The amount of cells left out are: ';

%creating comprehensable strings that are used in the.txt file
```

```
outDonor=([strDon, num2str(meanGenTimeDonor), strEnd, strAmountDon,
 num2str(amountDon),newline, strLeft, num2str(amountLeftDon)]);
outRecipient=([strRec, num2str(meanGenTimeRecipient),
 strEnd, strAmountRec, num2str(amountRec),newline, strLeft,
 num2str(amountLeftRec)]);
outTransconjugant=([strTra, num2str(meanGenTimeTransconjugant),
 strEnd, strAmountTra, num2str(amountTra),newline, strLeft,
 num2str(amountLeftTra)]);
allMeanValue=[outDonor, newline , outRecipient, newline,
 outTransconjugant, newline,newline, outCIDon, newline, outCIRec,
 newline, outCITra];
 %Save the confidence interval and mean division time into a .txt file
meanDivisionTimeAll =
 fullfile(expInfoObj.outputFolderPath,'meanDivisionTime.txt');
fprintf(fopen(meanDivisionTimeAll,'w'), allMeanValue);

%creating a histogram of the growthrates and also saving them
histOfAll=figure;
histogram(growthRateDonor, 'Normalization', 'pdf','DisplayName','Donors');
hold on
histogram(growthRateRecipient, 'Normalization', 'pdf','DisplayName','Recipients');
histogram(growthRateTransconjugant, 'Normalization', 'pdf','DisplayName','Transcon
legend('Donor', 'Recipient', 'Transconjugant');
title('Growth rate of donor, recipient and transconjugant cells');
ylabel('Relative frequency');
xlabel('Growth rate (min^{-1})');
outputFileName =
 fullfile(expInfoObj.outputFolderPath, 'growthrate_donor_recip_trans.fig');
savefig(outputFileName);
outputFileNamePNG =
 fullfile(expInfoObj.outputFolderPath, 'growthrate_donor_recip_trans.png');
exportgraphics(histOfAll,outputFileNamePNG)
hold off

%creating the plot for how many cells that are found in each frame
framesNumberOfCells=figure;
plot(phaseIdx, numberDonorsPerFrame);
hold on
plot(phaseIdx, numberRecipientsPerFrame);
plot(phaseIdx, numberTransconjugantsPerFrame);
legend('Donors', 'Recipients', 'Transconjugants');
title('Number of cells per frame for the three groups of cells');
xlabel('Frames');
ylabel('Number of cells');
outputFileNameFrames =
 fullfile(expInfoObj.outputFolderPath, 'number_of_cells_per_frame.fig');
savefig(outputFileNameFrames);
outputFileNameFramesPNG =
 fullfile(expInfoObj.outputFolderPath, 'number_of_cells_per_frame.png');
exportgraphics(framesNumberOfCells,outputFileNameFramesPNG)
hold off

%plotting the fluorescence of venus vs growthrate for each group
figure
```

```
plot(finalDonorYFPFluo, growthRateDonor, '*');
hold on
title('Fluorescence of YFP vs Growth rate donor');
xlabel('Fluorescence');
ylabel('Growth Rate');
plot(finalRecipientYFPFluo, growthRateRecipient, '.');
plot(finalTransconjugantYFPFluo, growthRateTransconjugant, 'p');
hold off
```



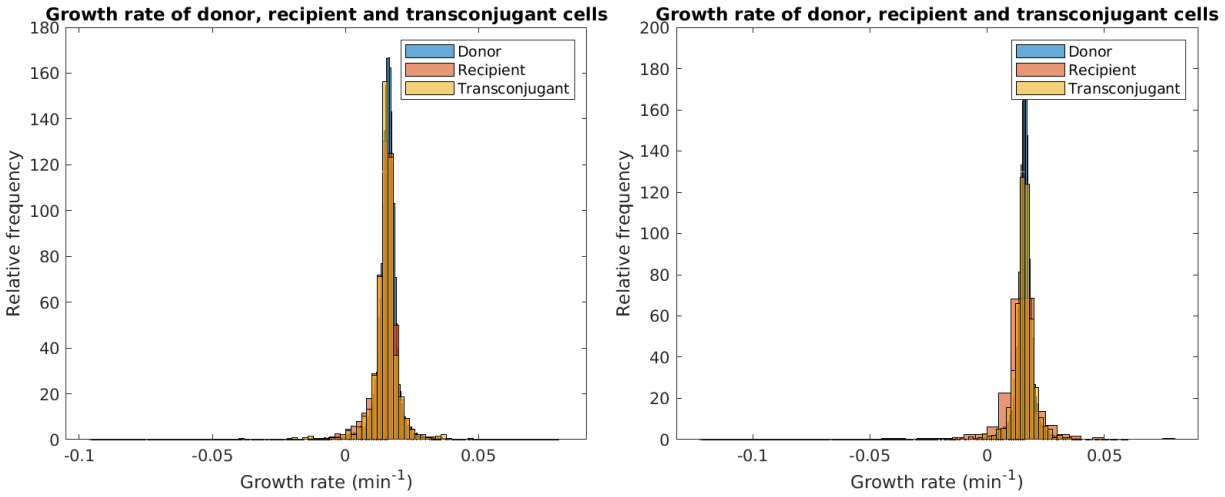

Growth rate of donor, recipient and transconjugant cells
Donor
Recipient
Transconjugant
Relative frequency
200
180
160
140
120
100
80
60
40
20
0
-0.15
-0.1
-0.05
0
0.05
Growth rate (min-1)


Number of cells per frame for the three groups of cells
Donors
Recipients
Transconjugants
Number of cells
2400
2200
2000
1800
1600
1400
1200
1000
800
600
0
50
100
150
200
250
Frames

E Growth Rate

The growth rate for the layer-loading experiments can be seen in the images below.

The growth rate for strain EL3336 and EL3354 can be seen in Figure 14.

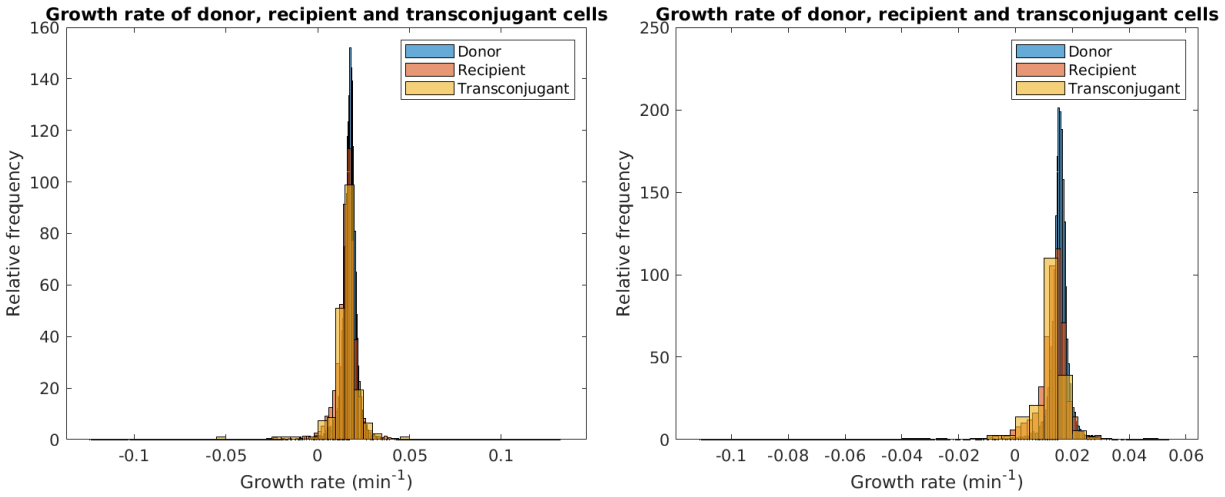


(a) The growth rate for the uninduced EL3336 and EL3354.

(b) The growth rate for the induced EL3336 and EL3354.

Figure 14: The growth rate for the induced and uninduced EL3336 donor cells together with the induced and uninduced EL3354 recipient cells. The EL3336 is seen as the blue parts (donors) in the histogram and the EL3354 are the orange (recipient) and yellow (transconjugant). For the induced cells, a shift can be seen regarding the growth rate of the recipient cells, these appear to have a slightly slower generation time.

The growth rate for strain EL3354 and EL3337 can seen in the figure below (Figure 15).

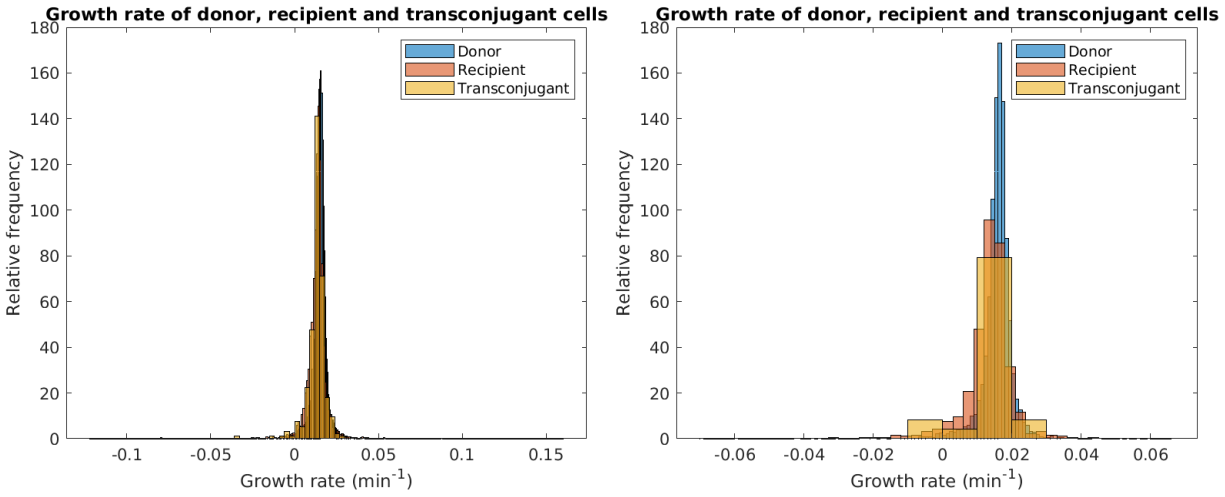


(a) The growth rate for the uninduced EL3337 and EL3354.

(b) The growth rate for the induced EL3337 and EL3354.

Figure 15: The growth rate for the induced and uninduced EL3337 donor cells and the induced and uninduced EL3354 recipient cells. The donor cells are blue in the histograms and the recipient cells are orange and the transconjugant cells can be seen as yellow. A slight shift can be seen for the recipient cells when they are induced, indicating that they have a slower growth rate than the donor cells.

The growth rate for strain EL3354 and EL3338 can be seen in Figure 16.

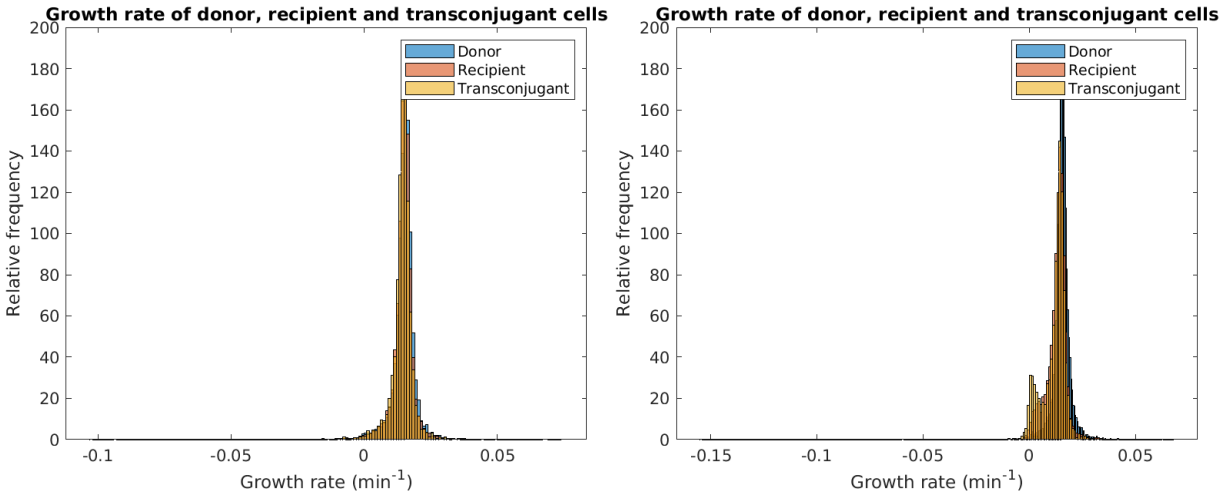


(a) The growth rate for the uninduced EL3338 and EL3354.

(b) The growth rate for the induced EL3338 and EL3354.

Figure 16: The growth rate for the induced and uninduced EL3338 donor (blue) cells and the induced and uninduced EL3354 recipient (orange) and transconjugant (yellow) cells. No significant shift can be seen in the growth rate for the donor cells and the recipient cells, by only looking at the histograms.

The histograms of the growth rates for the second run of the microfluidic experiments where the cells are mixed prior to loading them on the chip, which can be seen in the images below.

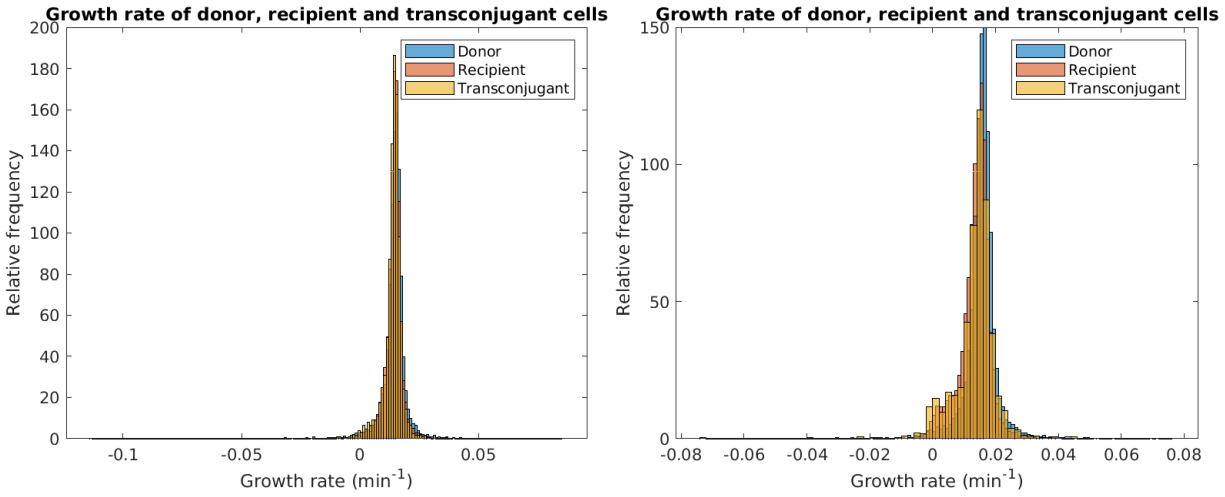


(a) The growth rate for the uninduced EL3336 and EL3354.

(b) The growth rate for the induced EL3336 and EL3354.

Figure 17: The growth rate for the induced and uninduced EL3336 donor cells together with the induced and uninduced EL3354 recipient cells. The EL3336 is seen as the blue parts (donors) in the histogram and the EL3354 are the orange (recipients) and yellow (transconjugants). For the induced cells, a shift can be seen regarding the growth rate of the recipient cells as well as a hump which gives two peaks of transconjugant cells.

The growth rate for strain EL3354 and EL3337 can seen in Figure 18.

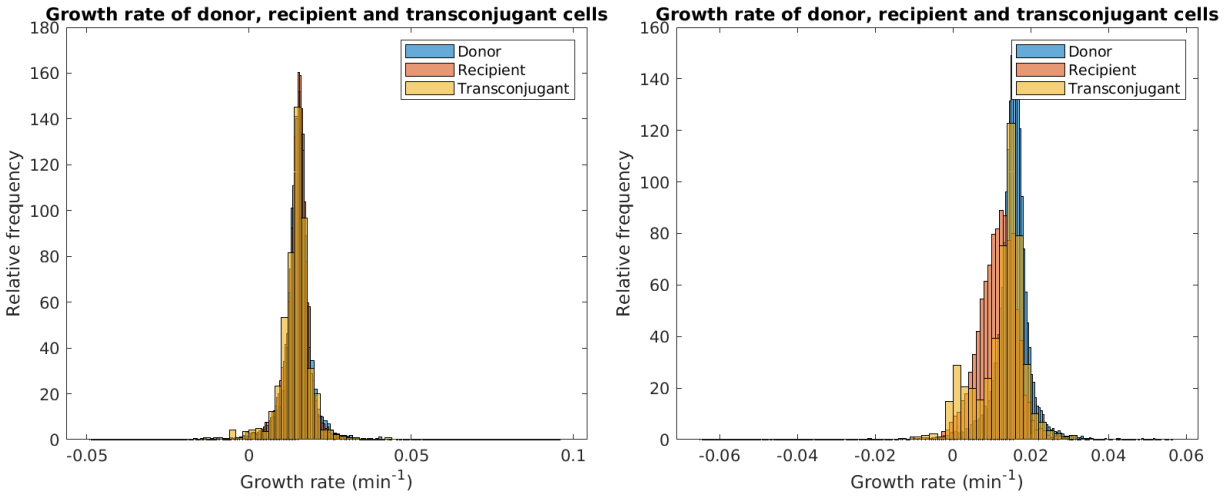


(a) The growth rate for the uninduced EL3337 and EL3354.

(b) The growth rate for the induced EL3337 and EL3354.

Figure 18: The growth rate for the induced and uninduced EL3337 donor cells and the induced and uninduced EL3354 recipient cells. The donor cells are blue in the histograms and the recipient cells are orange and the transconjugant cells are yellow. A slight shift can be seen for the transconjugant cells when they are induced. Indicating that they have a slower growth rate than the donor cells.

The growth rate for strain EL3354 and EL3338 can be seen in Figure 19.



(a) The growth rate for the uninduced EL3338 and EL3354.

(b) The growth rate for the induced EL3338 and EL3354.

Figure 19: The growth rate for the induced and uninduced EL3338 donor cells (blue) and the induced and uninduced EL3354 recipient (orange) and transconjugant (yellow) cells. A shift towards a slower growth rate can be seen for both recipient and transconjugant cells when they are induced. The transconjugant cells are almost creating two peaks in this histogram where the smaller peaks have values close to zero.