Master's Thesis

Abolishment of antibiotic resistance in *Escherichia Coli* using a conjugative CRISPR-Cas9 plasmid

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Emerging antibiotic resistance is one of the major threats to modern healthcare as well as to global food security and economic development. Approximately two-thirds of antibiotics administered to humans are ß-lactams. The emergence of extended-spectrum ß-lactamases (ESBLs) in a variety of bacteria confers multi-resistance to ß-lactams. In this study, an ESBL-harbouring pEC13 plasmid was targeted with a CRISPR-Cas9 plasmid that is delivered to the target *E. Coli* cells via conjugation machinery of the conjugative plasmid pLM2. An anti-ESBL plasmid was cloned, which carries an origin of transfer (o*riT*) domain for the conjugation initiation along with a gene for CRISPR-Cas9. One specific guiding RNA targeting the *IncFII* replication initiator gene of the pEC13 plasmid was designed and annealed to the anti-ESBL plasmid. In practice, the introduction of a modified anti-ESBL plasmid through a conjugation channel into an ESBL-harbouring bacterium leads to the expression of guiding RNAs that direct the Cas9 endonuclease to cleave the ESBL-plasmid, thus compromising its maintenance in the host. The results show that the induced anti-ESBL plasmid reduces the colony forming unit count of the ESBL-harbouring host by approximately two orders of magnitude on ß-lactam plates. The same ß-lactam concentration was tested to be lethal without the pEC13 resistance plasmid. Consequently, this is a viable proof-of-principle study, which shows that an anti-ESBL CRISPR-Cas9 plasmid can be introduced into ESBL-bacteria via conjugation, and its directed endonuclease activity can substantially hinder the survival of those ESBL-bacteria in the presence of lethal ß-lactam concentration.

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Kasvava antibioottiresistenssi on merkittävä ongelma, joka vaikuttaa globaalisti terveydenhoitoon, ruoantuotantoon ja taloudelliseen kasvuun. Beta-laktaamien antibioottiluokka kattaa noin kaksi kolmasosaa ihmisten käyttämistä antibiooteista. Laajan kirjon beta-laktamaasi (ESBL) -entsyymit mahdollistavat monipuolisen beta-laktaami-resistenssin useassa eri bakteerilajissa. Tässä tutkimuksessa kloonattiin konjugatiivinen CRISPR-Cas9-plasmidi, joka hyökkää pEC13 ESBL-plasmidia vastaan *E. Coli*-bakteerissa. Tutkimuksen CRISPR-Cas9-plasmidiin liitettiin *oriT (origin of transfer)* -sekvenssi, joka mahdollistaa tämän kokeellisen plasmidin konjugoimisen käyttäen toisen pLM2-plasmidin konjugaatiokoneistoa. CRISPR-Cas9-plasmidiin kohdentamiseksi pEC13-plasmidiin, siihen liitettiin myös *IncFII* crRNA-juoste. Tämän muokatun CRISPR-Cas9-plasmidin tulisi täten pystyä konjugoitumaan uuteen isäntäsoluun pLM2-plasmidin välityksellä, missä CRISPR-Cas9-plasmidin Cas9-endonukleaasi leikkaa pEC13-plasmidin DNA-kaksoisjuosteen sen *IncFII*-sekvenssin kohdalta. Leikatun pEC13-plasmidin ei tulisi enää selvitä isäntäsolussa, minkä tulisi johtaa isäntäsolun kuolemaan beta-laktaami-maljoilla. Tulokset osoittavat, että tutkimuksen CRISPR-Cas9 plasmidi konjugoituu uusiin isäntäsoluihin ja indusoitu CRISPR-Cas9-plasmidi vähentää beta-laktaamilla kasvavien isäntäbakteereiden määrää noin kahden kertaluokan verran. Kokonaisuutena, tätä tutkimusta voidaan pitää onnistuneena konseptin todistuksena, mikä näyttää, että kokeellinen CRISPR-Cas9-plasmidi on mahdollista konjugoida uuteen isäntäbakteeriin, missä sen CRISPR-Cas9-aktiivisuus huomattavasti heikentää isäntäbakteerien selviytymistä beta-laktaami-maljoilla.

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TERMS AND ABBREVIATIONS

TERMS

Agar	Solid growth medium from algae
CRISPR-Cas9	Molecular biology genome editing enzyme-complex
Fitness cost	Source for loss of fitness in an organism, usually external

ABBREVIATIONS

Cas9	CRISPR Associated Protein 9, endonuclease
Cfu	Colony Forming Unit
CRISPR	Clustered Regularly Interspace Short Palindromic Repeats
CRISPR-Cas9	Molecular biology genome editing tool
crRNA	CRISPR RNA
ESBL	Extended Spectrum Beta Lactamase
LB	Lysogeny Broth (Luria/Lennox Broth)
oriT	Origin of Transfer

1. INTRODUCTION

1.1 Growing antibiotic resistance

WHO (World Health Organization) has named the emerging antibiotic resistance as one of the major threats to global health, food security and economic development (WHO 2016). Antibiotic resistance generally means the distinct responses of pathogens to antimicrobial substances. Usually, the term is used in relation to bacteria, and their immunity to man-made antibiotics. The problem is truly global: reported rates of emergence of antibiotic resistance in common pathogenic bacteria are reaching new heights every year in all parts of the world. The pace of the change is accelerating, and some new untreatable strains have become resistant to our most potent antibiotics (such as carbapenems) in recent years (Laxminarayan *et al.* 2013). World Health Organization's GLASS program (Global Antimicrobial Resistance Surveillance System) concludes that the most commonly reported resistant bacteria were *Escherichia Coli* (e.g. ST131), *Staphylococcus aureus* (e.g. MRSA), *Klebsiella pneumoniae* and *Streptococcus pneumoniae* (WHO 2020). Many of these bacteria, such as methicillin-resistant *Staphylococcus aureus* (MRSA) regularly make the mainstream news and are now public knowledge.

Resistance is a consequence of mutations in microbial chromosomes and – increasingly – in transmissible extrachromosomal elements, e.g. plasmids. Suitable mutations combined with otherwise lethal antibiotic concentrations give a crucial competitive advantage to the mutated strains. Continued use of modern antibiotics in hospitals, agriculture and different communities has provided stable selection pressure for multiple multiresistant bacterial strains. The relative and perceived ineffectiveness of classical antibiotics has changed many prescription practices towards more modern, and more broad-spectrum antibiotics (such as ß-lactams), which further drives the evolution of new multiresistant strains. Furthermore, suboptimal antibiotic doses allow stepwise generation of resistance when a bacterial population contains many slightly varied strains with different antibiotic

susceptibilities. If antibiotic dosage is optimized to strains with the least susceptibility, bacterial ecology dictates that the more resistant strains will gain competitive advantage, leading to more rapid generation of resistant strains. Subinhibitory antibiotic concentrations have been shown to even enhance mechanisms relating to horizontal gene transfer, e.g. conjugation. The subinhibitory concentrations can also be regarded as antibiotic doses with subpar temporal length (Rice 2012).

1.2 Antibiotic resistance mechanisms

Alexander Fleming discovered penicillin in 1928, and first bacterial resistance mechanism – penicillinase - was identified in 1940, years before penicillin's full therapeutic introduction (Abraham and Chain 1940). Wide use of penicillin lead to prevalence of resistant strains, which lead to chemical modification of antibiotics. A similar action-reaction cycle of antibiotic development has continued to this day, although many novel antibiotic classes have been introduced since the 1940s.

After the discovery of penicillin and related penicillinase, humanity found itself in the midst of a billion-year war between pathogens and their hosts. The identification of first resistance mechanisms, and subsequent studies, revealed that the antibiotic r (resistance) genes are a naturally occurring part of microbial genome (Aminov and Mackie 2007). The evolutionary and ecological battle between microbial species had been raging since the dawn of life, and humanity just stumbled into it in the beginning of 20th century (Davies and Davies 2010).

The first ever discovered bacterial resistance mechanism, the penicillinase enzyme, belongs to a widely spread group of microbial ß-lactamases. ß-lactamases provide resistance to ß-lactam antibiotics by hydrolysing the namesake ß-lactam ring, thereby breaking it, and maiming the molecule (Blair *et al.* 2015). The heightened resistance against ß-lactams has been especially visible during the last decade. More than a 1000 varieties of ß-lactamases linked to antibiotic resistance have recently been identified. ESBLs, which confer resistance to multiple classes of ß-lactam

antibiotics, were first detected in a hospital setting in 1985. Since then, their prevalence has exploded, and their resistance profile widened. The first ESBLs against the last line of effective ß-lactams, the carbapenems, were already detected in 1993. These newest ESBLs, with carbapenemase activity, are now a major problem, and many so called superbacteria are a result of this (Davies and Davies 2010, Rice 2012)

1.3 Conjugative plasmids

Conjugation is the mechanism of transfer of DNA from one bacterial cell to another via specific molecular apparatus. It is one of the main mechanisms of horizontal gene transfer between bacteria. Conjugation occurs through cell-to-cell contact or through bridging pilus structure, and it always includes a donor (with the conjugative plasmid) and recipient bacterial cells. Conjugation is dependent on the properties of the transferring plasmid, and many naturally occurring plasmids can code for the conjugation apparatus, i.e. they are self-transmissible. Specific *tra* genes regulate the conjugation and related molecular machinery. A smaller part of them, the Dtr component, governs the processing of the plasmid DNA for transfer and the rest, Mpf component, is responsible for the formation of cell-to-cell linkage (Snyder *et al*. 2013).

Generally, conjugation starts when the time-wise downregulation of *Mpf* lifts, and a MPF starts to form between two cells. Distinct coupling proteins in the conjugation channel signal relaxase, which nicks the conjugative plasmid's origin-of-transfer (*oriT)* sequence and starts the transfer. Relaxase, bound to DNA, translocates to a recipient cell through a cell-cell opening or a pilus. Coupling proteins pump the whole DNA strand through to the recipient and relaxase seals the nick in the DNA. Both strands are replicated inside the donor and the recipient, yielding one intact plasmid inside each of the cells, leading to the closing of the conjugation channel. This process can be completely governed by the conjugative – self-transmissible – plasmid. A suitable plasmid with an *oriT* sequence can take advantage of pre-

existing conjugative plasmid's machinery to facilitate its own conjugation (Snyder *et al.* 2013).

In the 1950s Japanese researchers identified plasmids that could carry antibiotic resistance genes across the bacterial population using conjugation. Subsequently, this kind of horizontal gene transfer was found to be a fundamental property of bacterial populations. Research into plasmid-mediated transfer of antibiotic resistance has then received increased attention, because of its importance in the medical context (Davies and Davies 2010).

1.4 CRISPR-Cas system

CRISPR-Cas is an adaptive bacterial immune system. In its core is a specific RNA-guided endonuclease enzyme, which cleaves foreign genetic elements. The CRISPR loci in the bacterial genome are populated with distinct series of nucleotide repeats and spacers containing snippets of foreign DNA, cut from invading pathogens and mobile genetic elements. CRISPR loci are processed into small crRNA guide strands, which guide the CRISPR endonuclease. CRISPR-associated (*cas*) genes are located next to the repeat-spacer sequences, and they encode the protein machinery needed for crRNA processing and one or several endonuclease enzymes. During the processing of crRNAs, they are simultaneously loaded into the *cas*-coded endonuclease. This loading is mediated by the Cas-protein machinery and distinct endonucleases, depending on the specific CRISPR-Cas system. When the crRNA is processed and loaded into the multi-Cas protein complex, it will guide the cutting of complementary foreign DNA entering the cell. CRISPR system is hereditary and allows the generation of DNA-based memory from the invading pathogen's genetic material (Jiang *et al.* 2013).

The most widely used CRISPR system is the CRISPR-Cas9 system. Cas9 is a double-stranded DNA endonuclease, which uses the processed crRNA guides to specify the cleavage site. Cas9 belongs to the Type II CRISPR systems, as it only uses one type of large endonuclease to process the crRNAs and achieve double-strand break

of the target-DNA. In Type II systems, a special transactivating crRNA (tracrRNA) forms a complex with the crRNA, which RNA processing and formation of mature crRNA-tracrRNA Cas9 multi-protein complex (Wiedenheft *et al.* 2012). Cas9 requires base-pairing complementary between the crRNA and target DNA, and a short DNA motif called PAM (protospacer adjacent motif) immediately downstream from the target region. Type II CRISPR systems are unique because their target cleavage site can be programmed in the lab by introducing only a suitable tracrRNA:crRNA chimera to the cell. This ease-of-use of CRISPR-Cas9 system has allowed swift and easy genome editing in a multitude of organisms (Jinek *et al.* 2012).

1.5 Aims of the study

Prior to this study, Mattila *et al.* (2017) described how conjugative ESBL-plasmids from hospital pathogens can enable growth of other susceptible bacteria in the presence of lethal β-lactam concentrations. If this horizontal gene transfer phenomenon is able to rescue bacteria without inherent β-lactam resistance, it could be possible to invert the mechanism, and deliver a therapeutic plasmid to hospital pathogens by means of conjugation. A conjugative CRISPR-Cas9 plasmid with ESBL plasmid-crRNAs could conceivably be delivered to resistant pathogen strains via conjugation, where the Cas9 endonuclease would compromise the integrity of the ESBL-plasmid. This study seeks to clone and test such a conjugative CRISPR-Cas9 plasmid against previously described pEC13 ESBL plasmid (Mattila *et al.* 2017). The hypothesis was, that a suitable CRISPR-Cas9 plasmid with crRNA spacers against pEC13 can be cloned and transformed into a bacterial strain with a promiscuous (self-transmissible) pLM2 plasmid. Subsequently a CRISPR-Cas9 plasmid can be conjugated into pEC13 harbouring cells via the pLM2 conjugation machinery and the Cas9 enzyme can be induced to specifically attack the pEC13 plasmid in these recipient cells. Induced crRNA-Cas9 complex should target pEC13 sequence complementary to our incorporated crRNA, prompting a Cas9 endonuclease mediated double stranded cleavage of pEC13. Cleaved pEC13 should linearize and be degraded by the cellular enzymes, thus abolishing the inherent β-

lactamase activity. This should lead to negligible growth or death of the successful recipient cells in the presence of lethal β-lactam concentrations, which would not happen with intact pEC13. As a whole, this Master's Thesis was to be a proof-of-principle experiment, that would laid a groundwork for the future.

2. MATERIALS AND METHODS

The experimental work in this Master's Thesis was divided into three main parts: Plasmid cloning and preparation of the bacterial strains (Chapters 2.1, 2.2 and 2.3), Conjugation experiments (Chapter 2.4) and Induction experiments (Chapters 2.5 and 2.6).

2.1 Experimental plasmids

Our study had three main experimental plasmids, each with their own specific function (Table 1). pCas-oriT-IncFII was the main experimental plasmid, with CRISPR-Cas9 machinery and an anti-ESBL crRNA spacer (*IncFII* replication initiator gene from pEC13), and *oriT* sequence needed for conjugation. pLM2 plasmid was based on well-known promiscuous RP4 plasmid, and it should recognize the *oriT* sequence in our non-conjugative pCas9-oriT-IncFII plasmid and facilitate conjugation of both plasmids to a new host (Pansegrau, W. *et al.* 1988). The difference between RP4 and pLM2 was pLM2's amber mutations in amp^R and tet^R genes, thus rendering its host susceptible to corresponding antibiotics ampicillin and tetracycline. Previously isolated pEC13 was the target plasmid with *blaCTX-M-14* gene conferring β-lactam resistance to host cells (Mattila *et al.* 2017). CRISPR-Cas9 should target pEC13 with its *IncFII* crRNA.

Bacterial selection, screening and induction were accomplished with several different antibiotics. All the used antibiotics are assorted in Table 2. Listed antibiotic concentrations are the normal usage scenario concentrations, and that concentration of said antibiotic was used in all experiments, unless stated otherwise. All the work was done in *E. Coli* and therefore all the bacterial strains mentioned in this master's thesis are *E. Coli* strains (see Table 3 for main experimental strains).

Table 1. Experimental plasmids, including the main sequence features, presence of *tra* genes (self-transmissibility), innate antibiotic resistance genes and sequence reference.

Plasmid	Features	Self-trans-missible	Antibiotic resistance	Reference
pCas9-oriT-IncFII	CRISPR-Cas9 + *oriT* + *IncFII* crRNA sequence	No	Chloramphenicol	Addgene: Plasmid #42876
pLM2	Transfer (*tra)* genes for conjugation machinery	Yes	Kanamycin Tetracycline	RP4 plasmid: GenBank: BN000925.1
pEC13	*blaCTX-M-14* **E**xtended **S**pectrum **β L**actamase	Yes	Ampicillin and other ß-lactams	GenBank: KU932024.1

Table 2. Experimental antibiotics. All the used antibiotics and their normal usage (working) concentrations. These concentrations were used throughout the experiments, unless stated otherwise.

Name of the antibiotic	Working concentration
Ampicillin	100 µg/ml
Chloramphenicol	25 µg/ml
Kanamycin	25 µg/ml
Tetracycline	20 µg/ml
Rifampicin	100 µg/ml
Streptomycin	100 µg/ml

2.2 Molecular cloning

pCas9 plasmid (Addgene plasmid #42878) was a gift from Luciano Marraffini (Jiang *et al.* 2013). It is based on a pACYC184 backbone, and contains a tet promoter, which drivers the transcription of Cas9 gene from *S. pyogenes*. Downstream spacer sequence contains dual BsaI restriction sites in opposite directions, allowing scar-less cloning of new CRISPR spacers. Additionally, pCas9 contains a resistance gene for chloramphenicol.

oriT site (50980–51793 bps) was cloned from IncP RP4 plasmid (GenBank: BN000925.1, Pansegrau, Werner *et al.* 1994) using PCR and specific primers (Appendix 1). Phusion Hot Start II High Fidelity PCR Mastermix (Thermo Scientific, Waltham, MA, USA) containing necessary polymerase and oligonucleotides was used for the PCR reaction. *oriT* sequence was amplified using specific primers

(Appendix 1) from Metabion (Planegg, Germany) and a Pico 17 Thermal Cycler (Thermo Scientific) using a two-step PCR program (Appendix 2) with Phusion polymerase specific temperature steps.

SalI (Thermo Scientific) restriction enzyme was used to digest both pCas9 and amplified *oriT* sequence using a manufacturer's protocol. Digested pCas9 was dephosphorylated with FastAP phosphatase (Thermo Scientific) using a manufacturer's protocol.

oriT was ligated to pCas9 with a molar ratio of 5:1 using a manufacturer's protocol for T4 Ligase (Thermo Scientific). Cloned plasmid construct was transformed into competent DH5α cells using a standard heat shock transformation protocol: 10 µl of ligations (and calculated 50 ng of transformation plasmid controls) was combined with 100 µl of competent DH5α cells, aiming for optimal 1:10 DNA:cells volume. Mixed gently by pipetting 5 times. Incubated on ice for 30 min. Heat shocked the cells in 42 °C water for 30 s and added 900 µl of room temperature LB. Incubated 60 min (37 °C, shaking 220 rpm 37 °C, Excella E24, New Brunswick Scientific, Edison, NJ, USA). Cells were pelleted (2700 g, 5min) and resuspended to 100 µl of LB, diluted, plated on kanamycin LB agar plates (Table 2), and grown overnight (37 °C).

Successful transformants were selected with chloramphenicol LB agar plates and pure cultures were established from single transformant colonies and grown overnight (37 °C). After three subsequent pure cultures, two healthy colonies from distinct plate sectors were transferred to 5 ml liquid culture and grown overnight (37 °C). Plasmids were purified from 2 ml of bacterial culture using a Plasmid Spin Miniprep Kit (Qiagen, Hilden, Germany) with the manufacturer's protocol. Purified plasmids were digested with SalI (Thermo Scientific) and presence of separate *oriT* and pCas9 sequences was confirmed with AGE (0.7% Gel, 100V, 180 mA, 40 min).

Out of three possible crRNAs, one was selected to validate the experimental hypothesis. This crRNA, named IncFII, targets the namesake *IncFII* replication initiator gene in the pEC13 plasmid's sequence. Double stranded cleavage by the crRNA-Cas9 complex at the *IncFII* site linearizes the pEC13, which is then degraded

and unable to replicate, rendering its host susceptible to β-lactams (in our case, ampicillin).

IncFII sequence uniqueness was verified using Basic Local Alignment Search Tool (BLAST, Boratyn *et al.* 2013). To prevent any unintended cuts made by CRISPR-Cas9 *IncFII* sequence was ran against *E. Coli* K12 substr. MG1655 genome (NCBI Reference Sequence: NC_000913.3) and against RP4 plasmid sequence (GenBank: BN000925.1) in BLAST. BLAST yielded no hits, which proved that our *IncFII* crRNA sequence could be used in this experimental setting.

IncFII crRNA oligonucleotides (Appendix 1) – were synthetized by Sigma Aldrich (St Louis, MI, USA). Forward and reverse oligonucleotides were phosphorylated by the manufacturer, and they were annealed to each other according to pCas9 annealing instructions: 1 µl of both forward and reverse oligonucleotides (100 µM) were combined with 5 µl of 10X T4 Ligase buffer (New England Biolabs, Ipswich, MA, USA), 2.5 µl of 1 M NaCl and 40.5 µl of ddH_2O. Mixture was mixed by pipetting and spun briefly. Annealing was facilitated with a custom program in a T100 thermal cycler (Bio-Rad, Hercules, CA, USA), where the mixture was brought up to 95 °C for 5 min, and then cooled down to 20 °C in 1 °C/35 s increments. Afterwards, the annealed oligonucleotides were diluted 1:10, and stored in 4 °C.

pCas9-oriT was digested with BsaI (New England Biolabs) to generate complementary ends for the annealed *IncFII* oligonucleotides. The plasmid was also dephosphorylated with FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific) to ensure that the plasmid would not recircularize. Digested and dephosphorylated plasmid was run to an agarose gel with digestion and dephosphorylation controls. Upon validation of correct plasmid conformations from the gel, the correct experimental and control bands were incised and purified using QIAquick Gel Extraction Kit (Qiagen) with the manufacturer's instructions. Purified plasmids' concentrations were determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific).

Purified, digested and dephosphorylated pCas9-oriT was ligated to *IncFII* oligonucleotide following a standard protocol for T4 Ligase with the T4 Ligase buffer (New England Biolabs). However, due to extremely small size of the target-oligonucleotide insert (25 bp), 1 µl of insert was used. This corresponds to 24:1 molar insert:vector ratio, when 50 ng of pCas9-oriT vector was used. Ligation reaction was incubated 1.5 h in room temperature.

Complete pCas9-oriT-IncFII plasmid was then transformed into competent DH5α cells using a standard heat shock transformation protocol described previously. Transformants were plated to LB agar plates with chloramphenicol to allow screening of cells with pCas9 derived chloramphenicol resistance and subsequent pure cultures were made.

The complete pCas9-oriT-IncFII plasmid was verified using PCR with AGE. Specific primers were used for oriT and IncFII (Appendix 1) in two separate PCR runs. oriT sequence was detected with forward and reverse oriT primers, which should generate a 813 bp PCR product (oriT gene). IncFII sequence was detected with forward *IncFII* oligonucleotide with specifically designed reverse crRNA PCR probe, which should generate a 200 bp PCR product. PCR was run using Phusion Hot Start II High Fidelity PCR Mastermix (Thermo Scientific) like before, with plasmid and negative controls (see Appendix 2 for the PCR programs).

After PCR, both PCR products were run in 1% 200 ml AGE gel (with EtBr, 80 V, 180 mA, 60 min) and the presence of correct sized constructs (oriT and IncFII) was verified from the gel.

2.3 Bacterial strain preparation

After finished experimental plasmid cloning, the plasmids were conjugated and transformed into different *E. Coli* strains for distinct experimental parts of this Master's Thesis (Conjugation and Induction experiments). Conjugation experiments had a total of four main strains (one pair for 1st Phase conjugation and one pair for 2nd Phase Conjugation) and Induction experiment had one main strain.

Different strains were used in response to acquired results from the first experiments and to further optimize the conditions and antibiotic screens. Different strains exhibited different innate resistances to antibiotics and also provided a second viewpoint on the results. All the main experimental strains are assorted in Table 3.

Table 3. Main research bacterial strains, including their experimental function and antibiotic resistance derived from genomic DNA or experimental plasmids. All strains are *E. Coli* strains.

Experiment (Pair)	Strain name (plasmids)	Function	Antibiotic resistance (resistance plasmid)
1st Phase conjugation (Pair A)	Strain B BL21 Gold (pLM2)(pCas9-oriT-IncFII)	Pair A plasmid conjugation donor	Tetracycline Kanamycin (pLM2) Chloramphenicol (pCas9)
1st Phase conjugation (Pair A)	K-12 HMS174 Rif^R (pEC13)	Pair A plasmid conjugation recipient	Rifampicin Ampicillin (pEC13)
2nd Phase Conjugation (Pair B)	K-12 HB101 (pLM2)(pCas9-oriT-IncFII)	Pair B plasmid conjugation donor	Streptomycin Kanamycin (pLM2) Chloramphenicol (pCas9)
2nd Phase conjugation (Pair B)	Strain B BL21 Gold Rif^R (pEC13)	Pair B plasmid conjugation recipient	Tetracycline Rifampicin Ampicillin (pEC13)
Induction experiment	Strain B BL21 Gold (pEC13)(pCas9-oriT-IncFII)	Discrete induction experiments	Tetracycline Ampicillin (pEC13) Chloramphenicol (pCas9)

2.3.1 Bacterial strains for conjugation experiments

For conjugation experiments, two pairs of donor and recipient strains were used (Table 3). For first phase pair A, pLM2 plasmid was conjugated into BL21 Gold: BL21 Gold and HMS174 (pLM2) were both grown overnight (37 °C, shaking 220 rpm). The cells were washed once (2700 g, 5min) and 50 µl of each were combined, filled to 5 ml with LB. This conjugation culture was grown overnight, without antibiotics (37 °C, shaking 220 rpm). Transconjugants were selected with Kanamycin-Tetracycline plates and pure cultures were established. After four subsequent pure cultures, a singular pure culture colony of BL21 Gold (pLM2) was used to generate competent BL21 Gold (pLM2) cells: Singular colony 5 ml culture was grown overnight (37 °C, shaking 220 rpm) and it was diluted 1:50 to LB. This reseeded culture was grown 1 h 6 min (37 °C, shaking 200 rpm) until absorbance (at 550 nm) reached 0.269 (Ultrospec 10, Amersham Biosciences, Little Chalfont, United Kingdom), which was deemed indication for optimal exponential growth phase. Cells were chilled on ice for 20 min, and centrifuged (2800 g, 8 min, 4 °C). Supernatant was discarded and cells were resuspended to 12.5 ml of ice-cold 0.1 M $CaCl_2$. Cells were chilled again on ice for 20 min, centrifuged (2700 g, 8 min, 4 °C) and resuspended to 2.5 ml of ice-cold 0.1 M $CaCl_2$. Afterwards, cells were kept on ice for 24 h in a fridge (4 °C), and aliquoted as glycerol stocks (400 µl of competent cells and 100 µl of 50%/50% Glycerol-0.1 M $CaCl_2$).

pCas9-oriT-IncFII plasmid was purified using Plasmid Spin Miniprep Kit (Qiagen) and transformed into competent BL21 Gold (pLM2) cells using heat shock transformation described before (50 ng of DNA, 42 °C heat shock for 30 s). Successful transformants were screened with kanamycin-khloramphenicol plates and subsequent pure cultures were established. This BL21 Gold (pLM2)(pCas9-oriT-IncFII) strain served as the donor strain. Recipient strain was *E. Coli* HMS174 (pEC13) with a pEC13 ESBL plasmid from an earlier study (Mattila *et al.* 2017). This donor-recipient pair A was used for the first phase conjugation experiments (Table 3: Pair A).

For the second phase of the conjugation experiments, different donor-recipient pair B was used to allow different antibiotic selection screens (Table 3). The goal was to acquire HB101 (pLM2)(pCas9-oriT-IncFII strain), acting as the second phase conjugation donor.

First, pCas9-oriT-IncFII plasmid was purified Plasmid Spin Miniprep Kit (Qiagen) and electroporated to HMS174 (pLM2), which was already ready. HMS174 (pLM2) was grown overnight (37 °C, shaking 220 rpm). Cells were made electrocompentent by refreshing the normal 5 ml overnight culture as 1:50 dilution and then growing the cells (37 °C, shaking 220 rpm) until absorbance at 600 nm reached 0.30 (Ultrospec 10). This figure indicated optimal exponential growth phase. HMS174 (pLM2) culture was divided to separate microcentrifuge tubes for each electroporation reaction (1.4 ml) and kept on ice. Cells were centrifuged at (11519 g, 4 min, 4 °C), supernatants were discarded, and cells were resuspended to 1 ml of 4 °C ddH_2O. Centrifugation and resuspense step was repeated once again. Finally, the cells were centrifuged like before (11519 g, 4 min, 4 °C) and supernatant was carefully poured out (keeping residual left-over) and cells were resuspended to 20 µl of 4 °C ddH_2O. 2 µl of purified circular pCas9-oriT-IncFII plasmid DNA was added (80 ng and 8 ng (1:10 dilution) of plasmid DNA) and solution was mixed by pipetting. Solution was transferred to 1 mm electroporation cuvettes (BTX, Holliston, MA, USA) and electroporation was carried out in Gene Pulser Xcell (Bio-Rad) with 1,8 KV, 25 µF and 200 Ω parameters. Electroporated cells were resuspended to 950 µl L-broth and grown 1 h (37 °C, shaking 220 rpm). Cells were pelleted at 2700 g for 6 min, supernatant was discarded, and cells were resuspended to 100 µl of L-broth. Finally, successful transformants were selected with kanamycin-chloramphenicol LB agar plates and grown overnight (37 °C). Subsequent pure cultures were established, yielding successful HMS174 (pLM2)(pCas9-oriT-IncFII) clone.

To acquire the final HB101 (pLM2)(pCas9-oriT-IncFII) strain, both plasmids from HMS174 (pLM2)(pCas9-oriT-IncFII) were conjugated to competent HB101 cells. From each strain's pure cultures, one big colony was selected and scooped into 5 ml LB culture without antibiotics, which was grown 16.5 h (37 °C, shaking 220 rpm) to

facilitate conjugation. Successful transconjugants were selected with kanamycin-chloramphenicol-streptomycin plates and subsequent pure cultures were established. This resulted in successful second phase donor, HB101 (pLM2)(pCas9-oriT-IncFII) (Table 3).

For the second phase, recipient strain was also changed to BL21 Gold (pEC13). This strain was made rifampicin-resistant by cultivating the bacteria with spontaneously acquired rifampicin resistance. This was achieved by growing the strain in rising rifampicin concentrations for 5 consequent nights. The selected rifampicin concentrations were 5 µg/ml, 10 µg/ml, 20 µg/ml, 50 µg/ml and finally the normal rifampicin concentration 100 µg/ml. Ampicillin (full 100 µg/ml) was also present in each of the cultures to force pEC13 retainment in the cells. In each of the steps, 5 µl of previous culture was used to reseed the next 5 ml (filled to 5 ml with LB) culture. After these 5 consequent cultures, BL21 Gold Rif^R (pEC13) was successfully proven to be fully rifampicin resistant and served as the second phase conjugation recipient. This ready second pair (Table 3: Pair B) of donor-recipient strains was used in the second phase to further validate the conjugation ability of the experimental plasmid.

2.3.2 Bacterial strain preparation of induction experiments

For induction experiments, a one more strain was prepared to test just the Cas9 induction effects. The goal was to acquire a BL21 Gold (pCas9-oriT-IncFII)(pEC13) strain for the induction testing, which mimicked successful transconjugants, but without the pLM2 plasmid. HMS174 (pEC13) and BL21 Gold strains were grown overnight (37 °C, shaking 220 rpm). 100 µl of HMS174 (pEC13) culture was combined with 50 µl of BL21 Gold culture, filled to 3 ml with LB and grown overnight (37 °C, shaking 220 rpm) to facilitate plasmid conjugation. After the conjugation, transconjugants were selected with tetracycline-ampicillin plates. The conjugation strains were also cross tested for their antibiotic resistances, to make sure that only the transconjugant, BL21 Gold (pEC13), would survive on pure cultures. Three subsequent pure cultures were made to isolate a pure BL21 Gold

(pEC13) clone. pCas9-oriT-IncFII plasmid was purified using Plasmid Spin Miniprep Kit (Qiagen) and electroporated to BL21 Gold (pEC13), as described before. Successful transformants were selected with chloramphenicol-ampicillin plates. Subsequent three pure cultures resulted in a successful BL21 Gold (pCas9-oriT-IncFII)(pEC13) strain for the induction experiments, which was verified using PCR and AGE, similarly to before.

2.4 Plasmid conjugation

Multiple conjugation experiments were conducted, with differing parameters. Bacterial growth was assessed by screening the correct bacteria with functioning plasmids using antibiotic LB agar plates. Varying serial dilutions of bacterial cultures were made to LB, and these dilutions were plated to LB agar plates (unless stated otherwise). Colony forming unit (cfu) was used to estimate the number of bacteria in the experimental cultures, using the simple calculation:

$$cfu = \frac{number\ of\ colonies \times dilution\ factor}{plating\ volume} \quad (1)$$

All colony forming units are stated as cfu/ml. Number of colonies were counted by eye from the plates with countable colonies. Generally, the range for countable bacterial colonies is considered as 25-250 (Breed and Dotterrer 1916). Counts outside this 25-250 colony range were included for an estimate, if the colonies were still countable by eye and the other dilutions were uncountable. In out-of-range calculations, plates exceeding the 250 colonies were preferred over plates with very few colonies, as an error-minimizing mechanism. If multiple dilutions from the same series were in the 25-250 range, a simple average of these values was used. Confluent plates were mentioned if no numerical cfu value could be given. Dilution factor was the reciprocal of the actual dilution, e.g. dilution of 1:100, or $1:10^{-2}$, would have a dilution factor of 100, or 10^2. Eq. 1 was used for the cfu count throughout the results.

2.4.1 1st Phase conjugation

A total of four separate conjugation experiments were carried out with the pair A strains. This was done to test if the pCas9-oriT-IncFII was able to conjugate via the pLM2 conjugation machinery and if pEC13 in the recipient would affect this. Only two of these conjugation experiments yielded meaningful results and are described below:

In the first of these conjugation experiments, BL21 Gold (pLM2)(pCas9-oriT-IncFII) strain was used as the plasmid donor and HMS174 (pEC13) as the recipient. Additionally, the same HMS174 strain, without the pEC13 plasmid, was used as a recipient control strain. All strains were grown from pure cultures in 5 ml liquid cultures (16.00 h, 37 °C). The next day, all 5 ml cultures were washed twice (centrifuged, supernatant discarded and cells resuspended) with 5 ml LB (2714 g, 4 °C, 8 min). Each strain was plated to its corresponding antibiotic plates to determine the initial cfu count and grown overnight (37 °C). Four different conjugation cultures were established to 5 ml tubes without antibiotics, with 100 µl of both donor and recipient, filled to 3 ml with LB:

1. BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174 (Shaking, 220 rpm)
2. BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174
3. BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174 (pEC13) (Shaking, 220 rpm)
4. BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174 (pEC13)

All conjugation cultures were grown 3.0 hours in 37 °C, with or without shaking. The conjugation cultures were plated to LB, tetracycline, rifampicin, kanamycin-chloramphenicol and kanamycin-chloramphenicol-rifampicin -plates with varying dilutions (see Results) and grown overnight (37 °C).

In the second of these experiments, same donor and recipient strains were used. Additionally, BL21 Gold (pLM2)(pCas9) strain, with the unmodified pCas9 plasmid, was used as a donor control. Recipient control was not used. Otherwise, the same starting protocol was followed. Four different conjugation cultures were

established to 5ml tubes without antibiotics, with two different ratios of donor (D) and recipient (R), filled to 3 ml with LB:

1. BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174 (pEC13) (D:R: 100 µl:100 µl)
2. BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174 (pEC13) (D:R: 500 µl:5 µl)
3. BL21 Gold (pLM2)(pCas9) → HMS174 (pEC13) (D:R: 100 µl:100 µl)
4. BL21 Gold (pLM2)(pCas9) → HMS174 (pEC13) (D:R: 500 µl:5 µl)

All conjugation cultures were grown 3.0 h (37 °C, shaking 200 rpm) and plated to kanamycin-rifampicin-chloramphenicol plates (Table 2). Additionally, all conjugations were also plated to plates with diluted chloramphenicol concentrations, ranging from 1/4 dilution to 1/64 dilution (6.25 µg/ml to 0.39 µg/ml), with full amounts of kanamycin and rifampicin (See Results). All plates were grown overnight (37 °C).

2.4.2 2nd Phase conjugation

In the second phase, new bacterial strains were used to allow different antibiotic selection screens on plates (Table 3). Experimental setup was similar to the first phase, but some changes were made to how the donor was grown. HB101 (pLM2)(pCas9-oriT-IncFII) was the donor and BL21 Gold Rif^R (pEC13) was the recipient, hence the only conjugation in the 2nd phase was:

HB101 (pLM2)(pCas9-oriT-IncFII) → BL21 Gold Rif^R (pEC13)

In the first conjugation experiment, both the donor and the recipient were grown 16.00 h (37 °C, shaking 220 rpm). Starting cell count plates were made from both strains. Donor was then refreshed as 1:1000 dilution (25 µl culture/25 ml LB) with normal concentration kanamycin-chloramphenicol (to force plasmid retainment) and grown (37 °C, shaking 220 rpm). The recipient was kept on ice. At two hours donor growth-time, 1 ml from both strains was washed twice (6800 g, 5min) and 2 h donor cell count plates were made. Two conjugation cultures were established, with 50 µl/50 µl and 50 µl/5 µl donor/recipient ratios, filled to 3 ml with LB.

Conjugation cultures were grown for 2.0 h (37 °C, shaking 220 rpm). At five hours of donor growth time, the exact same steps were followed as in two-hour timepoint, but only 50 µl/50 µl conjugation was established. All conjugation cultures were plated to transconjugants selecting plates and grown overnight (37 °C).

In the second experiment, the same strains were used in a simpler setup. Both the donor and the recipient were grown 16.25 h (37 °C, shaking 220 rpm) and washed (6800g, 5min). Starting cell count plates were made. Donor was not reseeded, like earlier. Two conjugation cultures were established with 50 µl/50 µl and 50 µl/5 µl donor/recipient ratios, filled to 3 ml with LB. Conjugation cultures were grown 2 h 35 min and only transconjugants were plated to chloramphenicol-rifampicin plates, which were grown overnight (37 °C).

2.5 Minimum inhibitory concentration

During the different experiments, we noticed some poor and abnormal growth patterns in the experimental bacteria, when subjected to multiple antibiotics. Heavy antibiotic load could feasibly hinder the growth of otherwise viable bacteria. Minimum inhibitory concentration (MIC) test was carried out to obtain an approximate for a minimum antibiotic concentration which starts to impair growth on a naïve strain. Tetracycline and chloramphenicol dilution series were tested on HMS174 (pEC13), which does not have a resistance genes for either of them. A growth curve experiment for approximating the minimum inhibitory concentration was devised.

HMS174 (pEC13) was grown 16.00 h in a 5 ml culture and washed once (2714 g, 4 °C, 8 min), then stored on ice. The cells were diluted and 5 µl of cells was combined with 1.0 ml of diluted antibiotics, bringing the total volume to 1005 µl. This yielded cell count of 1.59×10^4 cfu/ml in the final volume. 10^4 cfu/ml was in the optimal range for this kind of experiment. The normal usage concentration of tetracycline would be 20 µg/ml and the following tetracycline dilutions were made to LB (final antibiotic concentration in parentheses): 1:100 (199.0 ng/ml), 1:200 (99.5 ng/ml),

1:300 (66.5 ng/ml), 1:400 (49.8 ng/ml), 1:450 (44.2 ng/ml), 1:500 (39.8 ng/ml), 1:600 (33.2 ng/ml), 1:650 (30.6 ng/ml), 1:700 (28.3 ng/ml), 1:800 (24.9 ng/ml), 1:900 (22.3 ng/ml) and 1:1000 (19.9 ng/ml). The normal usage concentration of chloramphenicol would be 25 µg/ml (Table 2) and the following chloramphenicol dilutions were made to LB (final antibiotic concentration in parentheses): 1:40 (621.9 ng/ml), 1:50 (497.5 ng/ml), 1:60 (414.8 ng/ml), 1:70 (355.1 ng/ml), 1:80 (310.9 ng/ml), 1:90 (276.1 ng/ml), 1:100 (248.8 ng/ml), 1:150 (165.4 ng/ml), 1:200 (124.4 ng/ml) and 1:250 (99.5 ng/ml).

Each mixture of cells and diluted antibiotics were thoroughly vortexed and pipetted to Honeycomb 2 microplate wells (Oy Growth Curves Ab Ltd, Helsinki, Finland) as triplicates. This yielded 36 samples for tetracycline and 30 samples for chloramphenicol. Same diluted HMS174 (pEC13) cells, without any antibiotics, and pure LB were used as control samples, also in triplicate. Growth curve experiment was conducted in Bioscreen C MBR (Oy Growth Curves Ab Ltd), with the following parameters: Shaking setting "Low" (stop for 5s before measurement), absorbance measurement at 580 nm every 10 minutes, 37 °C, 24,00h. The raw data was analysed and graphed in Excel 2016 (Office 2016, Microsoft, Redmond, NM, United States).

2.6 Induction of Cas9

A dedicated experiment for testing the effects of Cas9 induction on cell growth was devised. BL21 Gold (pEC13)(pCas9-oriT-IncFII) (Chapter 2, Table 3) was prepared and used as a model transconjugant strain, which had both the pEC13 and pCas9-oriT-IncFII plasmids, but no pLM2 plasmid. Transconjugants from conjugation experiments described above would have all the three experimental plasmids: pEC13, pCas9-oriT-IncFII and pLM2. This dedicated induction experiment was simpler (without pLM2) and allowed less usage of antibiotics, as it had proved problematic to grow bacteria with several antibiotics to force plasmid retainment. As mentioned, Cas9 machinery is driven by the *tet* promoter, which was induced using a full tetracycline concentration (20 µg/ml) in a liquid culture. The raw data

was analysed and graphed in Excel 2016 (Office 16, Microsoft) and is presented in Chapter 3.

2.6.1 1st Induction experiment

The dedicated induction experiment strain (Table 3) BL21 Gold (pEC13)(pCas9-oriT-IncFII) was grown 16.00 h (37 °C, shaking 220 rpm) and was washed twice with LB (2714 g, 4 °C, 8 min). Washed cells were used to establish starting cell count plates. Induction was carried out by combining 1.5 ml of washed BL21 Gold (pEC13)(pCas9-oriT-IncFII) culture with 1.5 ml of LB, which was grown 3.0 h (37 °C, shaking 220 rpm) in the presence of full (20 µg/ml) tetracycline. Identical uninduced control sample was established simultaneously, without inducing tetracycline. After induction, the samples were plated to Ampicillin, Ampicillin-Tetracycline, Ampicillin-Chlroamphenicol and LB -plates and grown overnight (37 °C).

2.6.2 2nd Induction experiment

After the first induction experiment, some adjustments to the bacterial plating dilutions were needed, to establish the cfu/ml count more accurately. Otherwise, the experiment was conducted in an identical manner to the first induction experiment.

2.6.3 Induction experiment without pEC13

One similar induction experiment was conducted without the target pEC13 plasmid, to understand how Cas9 induction without the target pEC13 would affect the growth. BL21 Gold (pLM2)(pCas9-oriT-IncFII) strain (Table 3) was grown for 16.00 h (37 °C, shaking 220 rpm). The cells were washed twice (2700 g, 4 °C, 8 min) and starting cell count plates were made. 750 µl of bacterial culture was combined with 750 µl of LB and grown 3.0 h (37 °C, shaking 220 rpm) in the presence of full tetracycline concentration (20 µg/ml). For a control, identical uninduced culture

was made without tetracycline. Induced and uninduced samples were plated on chloramphenicol-kanamycin and chloramphenicol-tetracycline plates and grown overnight (37 °C).

2.6.4 Induction experiment without pCas9

In this last induction experiment, multiple concentrations of tetracycline were used to understand the effects of tetracycline induction protocol on cell growth. For this experiment, BL21 Gold (pEC13) was used, as it allowed approximating the effects of induction protocol on cell growth, without inducible Cas9. BL21 Gold strains were resistant to tetracycline (Table 3), but tetracycline would still affect growth. Like before, BL21 Gold (pEC13) was grown 16.00 h (37 °C, shaking 220 rpm) and starting cell count plates were plated. Cells were washed twice with LB (2714 g, 4 °C, 8 min). For the induction, 750 µl of bacterial culture was combined with 750 µl of LB and grown 3.0 h (37 °C, shaking 220 rpm) in the presence of full (20 µg/ml), 1:10 (2.0 µg/ml) and 1:100 (200 ng/ml) tetracycline. One identical culture was established as the uninduced control, without tetracycline. Induction cultures were plated on Ampicillin-Tetracycline and LB -plates and grown o/n (37 °C).

3. RESULTS

Results are divided according to the Chapter 2 order. See Table 2 for the normal usage antibiotic concentrations and Table 3 for the main experimental strains. See Eq. 1 for the cfu calculations and related colony counting considerations (Chapter 2.4).

3.1 Plasmid conjugation

Plasmid conjugation experiment results are presented as tables, as graphical presentation would hide some of the details in the results. Conjugation frequency (or efficiency) can be calculated as the relationship of transconjugants concentration to donor concentration, or as the relationship of transconjugants concentration to total cell concentration. As total cell concentration is usually defined as a sum of donor, recipients and transconjugants concentrations (Headd and Bradford 2020), this could not be applied to all of our conjugation experiments, thus the conjugation frequency was determined using Eq. 2:

$$Conjugation\ frequency = \frac{transconjugant\ cell\ concentration}{donor\ cell\ concentration} \qquad (2)$$

As transconjugant and donor cell concentrations are calculated as cfu/ml, the conjugation frequency is unitless. Higher conjugation frequency figure implies more optimized conjugation between the donors and recipients.

In conjugation experiments, we ran to a problem with abnormally small transconjugant colonies with aberrant morphology (see Discussion). Most of the time, it was still possible to count these aberrant colonies. For clarity, in all tables, cfu counts marked with an asterisk (*) specify plates with abnormally small, but countable, colonies.

3.1.1 1st Phase conjugation

In this phase, the main conjugation strains were BL21 Gold (pLM2)(pCas9-oriT-IncFII) as the donor and HMS174 (pEC13) as the recipient. Main experimental conjugation was:

BL21 Gold (pLM2)(pCas9-oriT-IncFII) → HMS174 (pEC13)

In the first conjugation experiment of the first phase, differing conjugation parameters were tested by establishing four conjugation cultures, with or without shaking (220 rpm) and with two different recipients (Table 4). Donor in every culture was BL21 Gold (pLM2)(pCas9-oriT-IncFII) and the donor to recipient ratio 1:1.

Table 4. Calculated cfu counts of the first conjugation experiment in the first phase. Cfu counts marked with an asterisk (*) specify plates with abnormally small, but countable, colonies.

Screened strain (antibiotic plate)	Recipient: HMS174 Shaking	Recipient: HMS174	Recipient: HMS174 (pEC13) Shaking	Recipient: HMS174 (pEC13)
Total cell count (No antibiotics, pure LB)	2.32×10^9	7.6×10^8	2.64×10^9	5.1×10^8
Donor (Tetracycline)	1.12×10^8	1.0×10^6	5.5×10^8	9.0×10^5
Recipient (Rifampicin)	9.2×10^8	1.89×10^8	4.6×10^8	1.6×10^8
Strains with pLM2 and pCas9 plasmids (Kanamycin-Chloramphenicol)	100 % Confluent (10^2)	85 % confluent (10^2)	95 % confluent (10^2)	90 % confluent (10^2)
Recipient transconjugants (Kanamycin-Rifampicin-Chloramphenicol)	110 *	90 *	1.0×10^4	360 *
Conjugation frequency	9.2×10^{-7}	$9{,}00 \times 10^{-5}$	$1{,}82 \times 10^{-5}$	$4{,}00 \times 10^{-4}$

Table 4 has screened strains on the right, with the antibiotics used in screening. Confluency percentage provided as an approximate estimate with the dilution factor in parentheses. The strains had the following pre-conjugation starting cfu counts: BL21 Gold (pLM2)(pCas9-oriT-IncFII): 8.8×10^8, HMS174: 5.0×10^8 and HMS174 (pEC13): 2.09×10^9. Conjugation frequency varied within three orders of magnitude, even when pre-conjugation donor and recipient cfu counts were in the similar and expected range. Shaking incubator yielded higher cfu counts (roughly one order of magnitude) on both HMS174 and HMS174 (pEC13) transconjugants, as well as in total cell counts.

In the second conjugation of the first phase, two donors with differing plasmids were used with the same recipient, HMS174 (pEC13). Additionally, two different donor to recipient ratios were used and antibiotic plate selection was done only for transconjugants (kanamycin-rifampicin-chloramphenicol) with chloramphenicol dilution series (Table 5).

Table 5. Calculated cfu counts of the second conjugation experiment in the first phase. Cfu counts marked with an asterisk (*) specify plates with abnormally small, but countable, colonies.

Antibiotic plate	Donor: BL21 Gold (pLM2) (pCas9-oriT-IncFII) (Ratio 1:1)	Donor: BL21 Gold (pLM2) (pCas9-oriT-IncFII) (Ratio 100:1)	Donor: BL21 Gold (pLM2) (pCas9) (Ratio 1:1)	Donor: BL21 Gold (pLM2) (pCas9) (Ratio 100:1)
Kanamycin-Rifampicin-Chloramphenicol	8.3×10^2 *	2.7×10^2	0	0
Kanamycin-Rifampicin-$\frac{1}{4}$ Chloramphenicol	6.94×10^3	7.9×10^2	0	4.0×10^2 *
Kanamycin-Rifampicin-$\frac{1}{16}$ Chloramphenicol	Uncountable	2.79×10^3	1.2×10^4 *	8.16×10^3
Kanamycin-Rifampicin-$\frac{1}{32}$ Chloramphenicol	8.0×10^3 *	2.60×10^3	2.45×10^5	2.0×10^4
Kanamycin-Rifampicin-$\frac{1}{64}$ Chloramphenicol	2.4×10^4	2.66×10^3	2.76×10^5	2.4×10^4

In the second experiment of the first phase, donor to recipient ratio of 1:1 yielded higher transconjugant count (roughly one order of magnitude) than the ratio of

1:100 across all samples (Table 5). The strains had the following pre-conjugation starting cfu counts: BL21 Gold (pLM2)(pCas9-oriT-IncFII): 6.6×10^8, BL21 Gold (pLM2)(pCas9): 8.6×10^8 and HMS174 (pEC13): 5.8×10^8. Transconjugants plates were empty for the unmodified (untransmissible) pCas9 plasmid strain in the higher chloramphenicol concentrations, but decreasing chloramphenicol concentration yielded screened transconjugants, which may not be true transconjugants, but survived recipients (see Discussion).

3.1.2 2nd Phase conjugation

In this phase, the main conjugation strains were HB101 (pLM2)(pCas9-oriT-IncFII) as the donor and BL21 Gold Rif^R (pEC13) as the recipient. Main experimental conjugation was:

HB101 (pLM2)(pCas9-oriT-IncFII) → BL21 Gold Rif^R (pEC13)

Contrary to the first phase conjugation, donor cfu counts were not obtained after the conjugation, from the conjugation culture itself, but before the conjugation experiment. Generally, the whole conjugation culture is sampled for the donors, recipients and transconjugants (Headd and Bradford 2020), as was done in first phase. Short conjugation times for the second phase (2.0 and 2.5 h) still allow approximation of the conjugation frequency from starting donor cfu, but this frequency is not directly comparable with the first phase.

In the first conjugation experiment of the second phase, the only one conjugation strain pair was used, as stated above (all conjugation pairs in Table 3). The effect of donor growth phase on conjugation frequency was examined with two donor growth timepoints and with two different donor to recipient ratios (Table 6).

Table 6. Calculated cfu counts of the first conjugation experiment in the second phase. Cfu counts marked with an asterisk (*) specify plates with abnormally small, but countable, colonies.

Screened strain (Antibiotic plate)	Donor grown 2.0 h	Donor grown 5.0 h
Donor (Kanamycin-Chloramphenicol)	4.8×10^6	9.0×10^7
Recipient transconjugants 1:1 ratio (Rifampicin-Chloramphenicol)	2.0×10^3 *	1.10×10^3 *
Recipient transconjugants 10:1 ratio (Rifampicin-Chloramphenicol)	1.26×10^3 *	N/A
Conjugation frequency 1:1 ratio	4.17×10^{-4}	1.22×10^{-5}
Conjugation frequency 10:1 ratio	2.63×10^{-4}	N/A

In table 6, 1:10 ratio for the 5 h donor growth time point was not obtained. The strains had the following starting cfu counts: HB101 (pLM2)(pCas9-oriT-IncFII): 5.2×10^8 and BL21 Gold Rif^R (pEC13): 1.31×10^7, which were in line with expectations. Conjugation frequency slightly favoured donor to recipient ratio of 1:1, and conjugation frequencies were among the best yet seen, although they are not directly comparable with first phase conjugation experiments (see Discussion).

In the second conjugation experiment of the second phase the donor and the recipient strains were the same as in the first experiment (with ratios 1:1 and 1:10), but donor was grown 16.25 h and was not reseeded (Table 7).

Table 7. Calculated cfu counts of the second conjugation experiment in the second phase. Cfu counts marked with an asterisk (*) specify plates with abnormally small, but countable, colonies.

Screened strain (Antibiotic plate)	Donor grown 16.25 h
Donor (Kanamycin-Chloramphenicol)	8.0×10^8
Recipient transconjugants 1:1 ratio (Rifampicin-Chloramphenicol)	1.5×10^5 * (approximate)
Recipient transconjugants 10:1 ratio (Rifampicin-Chloramphenicol)	1.18×10^4 *
Conjugation frequency 1:1 ratio	1.88×10^{-4} (approximate)
Conjugation frequency 10:1 ratio	1.48×10^{-5}

Approximation in the form of extrapolation had to be used for the 1:1 ratio conjugation cfu counts, as no plate had precisely countable colonies, and this is reflected on the conjugation frequency calculation as well (Table 7). The starting cfu counts were: HB101 (pLM2)(pCas9-oriT-IncFII): 8.0×10^8 and BL21 Gold Rif^R (pEC13): 2.5×10^8. Conjugation frequency is similar to the earlier conjugation experiment, even with approximations. Longer donor growth time (without reseed) does not significantly raise the conjugation frequency (Table 7), although the absolute amount of transconjugants is one order of magnitude higher than in the first experiment of the second phase (Table 6).

3.2 Minimum inhibitory concentration

The non-lethal mechanism of action of tetracycline was examined with a tetracycline-naïve HMS174 (pEC13) strain growth curve experiment (Figure 2).

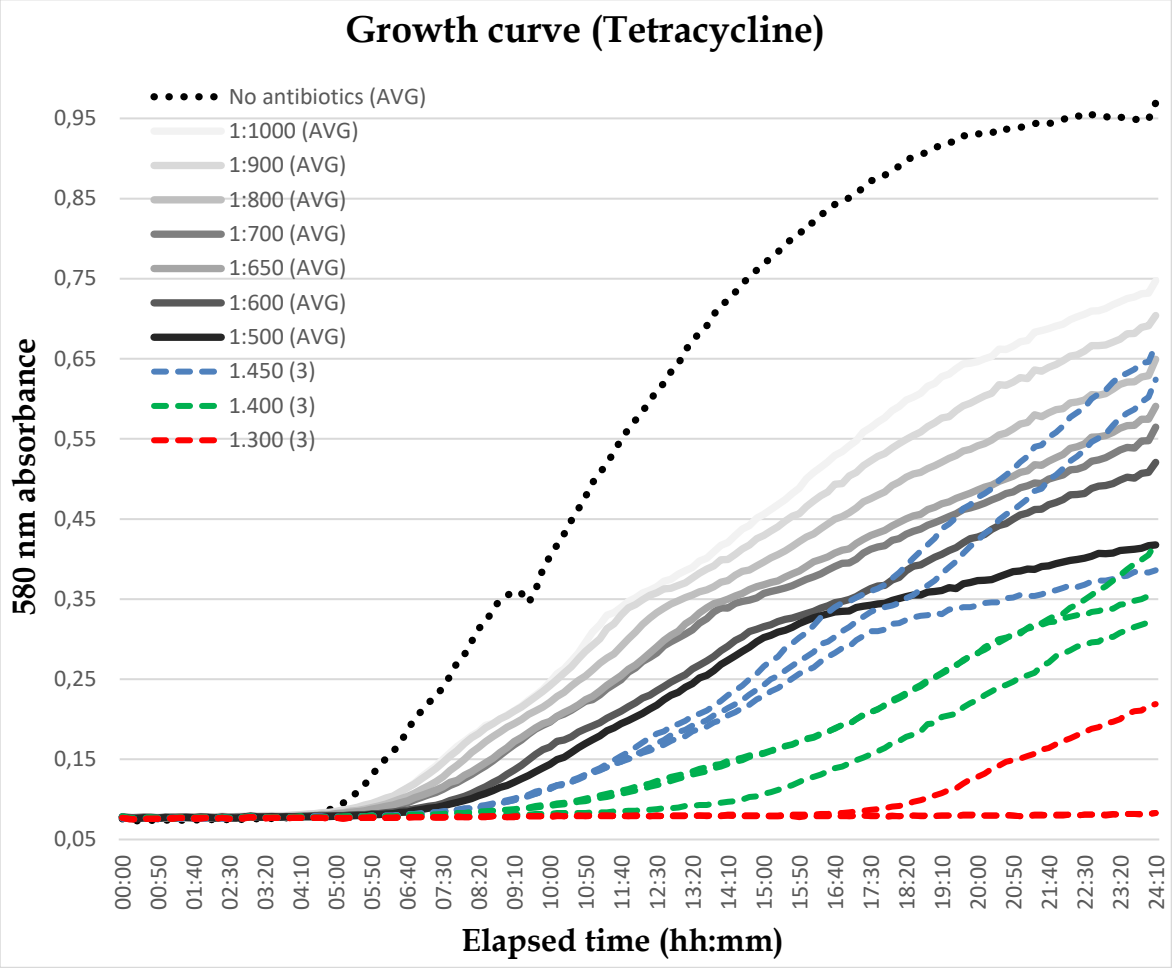

Figure 2. Growth curve analysis for approximating the minimum inhibitory concentration of tetracycline. Measured absorbance (580 nm) on the y-axis, time (hours:minutes) on x-axis. Simple average (AVG) from triplicate samples for each dilution was calculated (solid grayscale lines), except for dilutions 1:300, 1:400 and 1:450 (colourful dash lines), in which each triplicate is depicted individually (marked with (3)). Dilutions 1:100 and 1:200 did not grow above the baseline and are not depicted. Other controls than "No antibiotics" (black dotted line) are not depicted.

Tetracycline minimum inhibitory concentration growth curve demonstrated some inconsistencies among its dilutions (Figure 6). Dilutions 1:300, 1:400 and 1:450 exhibited more variance in their growth and were not averaged (dashed lines). In 1:300 dilution, only one of the triplicates (red dashed line) grew above the baseline, and other two were at the baseline. The certain minimum inhibitory concentration

for tetracycline, with a naïve strain, can be considered to be 1:200 dilution (99.5 ng/ml).

The mechanism of action is similar between tetracycline and chloramphenicol, and also chloramphenicol growth curves were examined with the same a naïve HMS174 (pEC13) strain (Figure 3).

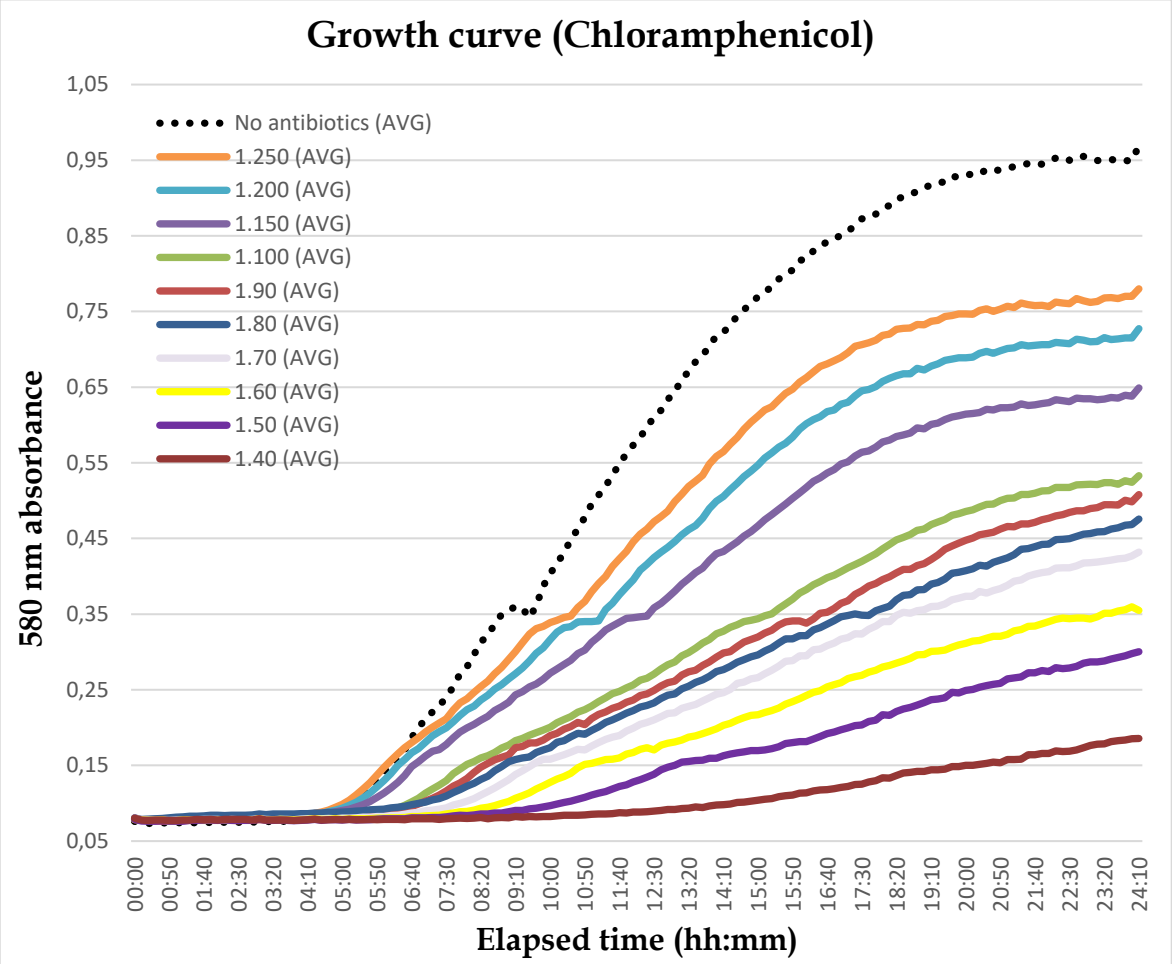

Figure 3. Growth curve analysis for approximating the minimum inhibitory concentration of chloramphenicol. Measured absorbance (580 nm) on the y-axis, time (hours:minutes) on x-axis. Simple average (AVG) from triplicate samples for each dilution was calculated (solid-coloured lines). Other controls than "No antibiotics" (black dotted line) are not depicted.

Chloramphenicol demonstrated more regular growth curves (Figure 3), compared with tetracycline (Figure 2).The growth curves were in the expected order, with lower chloramphenicol concentrations allowing higher amount of bacteria to flourish. The relationship between the chloramphenicol concentration and growth is moderately linear, as can be seen from the even spacing of the growth curves in

Figure 3. No clear minimum inhibitory concentration for chloramphenicol could be acquired, as HMS174 (pEC13) grows readily, even with the highest concentration 622 ng/ml (1:40 dilution), which was unexpected. Both antibiotics exhibited similar growth lag phase, with the absorption starting to rise at the 5-hour mark (Figure 2, Figure 3).

3.3 Induction of Cas9

A total of four induction experiments were performed, where tetracycline was used to activate Cas9's *tet* promoter. First two experiments were carried out with the dedicated induction strain BL21 Gold (pEC13)(pCas9-oriT-IncFII) (Figure 4, Figure 5), to understand how Cas9 activation would affect the host's growth on ampicillin (β-lactam), with differing plasmid selecting antibiotic pairs.

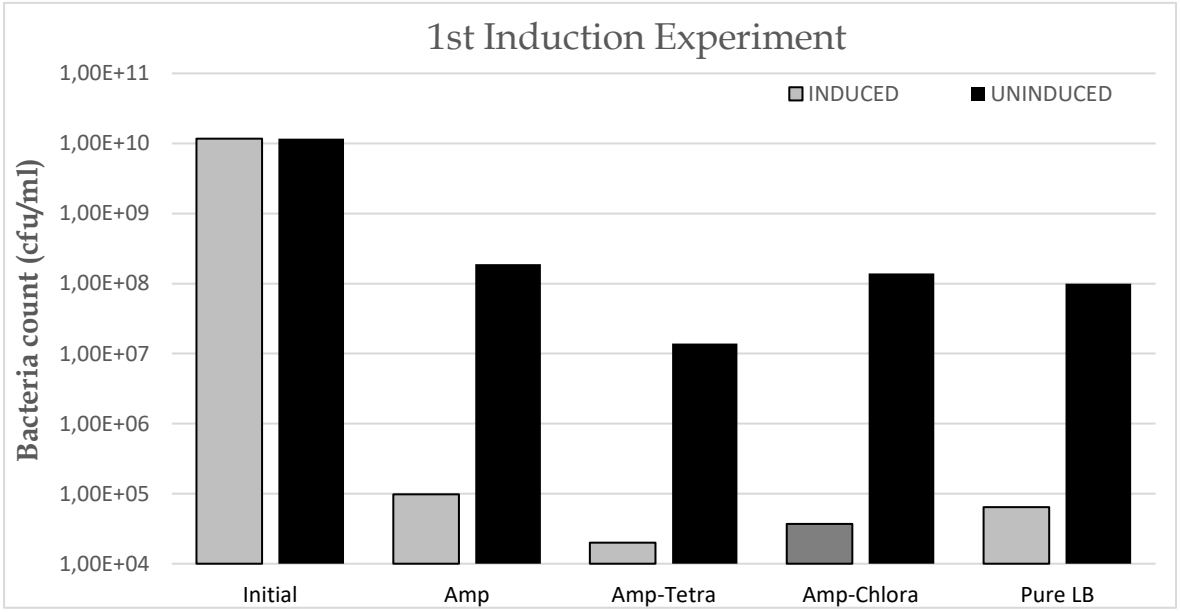

Figure 4. Calculated cfu counts for the first induction experiment. Induction cultures were plated on depicted antibiotic LB agar plates. Calculated cfu/ml on the logarithmic y-axis, which is cut at the 10^4 gridline. Antibiotic abbreviations on x-axis: Ampicillin (Amp), Tetracycline (Tetra), Chloramphenicol (Chlora). Pure LB means a normal LB agar plate without any antibiotics. Induced samples in grey, uninduced in black. Distinct dark-grey colour in especially interesting Ampicillin-Chloramphenicol induced sample.

First induction experiment results were partially in line with the prior expectations: induction starkly lowered the cfu counts on all plates with ampicillin, as Cas9-mediated pEC13 cleaving would cause (Figure 4). But induction also lowered cell

counts on plates without antibiotics, which led to better understanding about secondary mechanisms hindering cell growth (see Discussion). Induction protocol involved diluting the cells 1:1 to LB, which partly explains the lower cfu counts in the uninduced samples when compared with initial counts, as was the case in every induction experiment. The ampicillin-tetracycline plates would have continued to induce Cas9 even on the plates, which yielded the lowest cfu counts of this experiment.

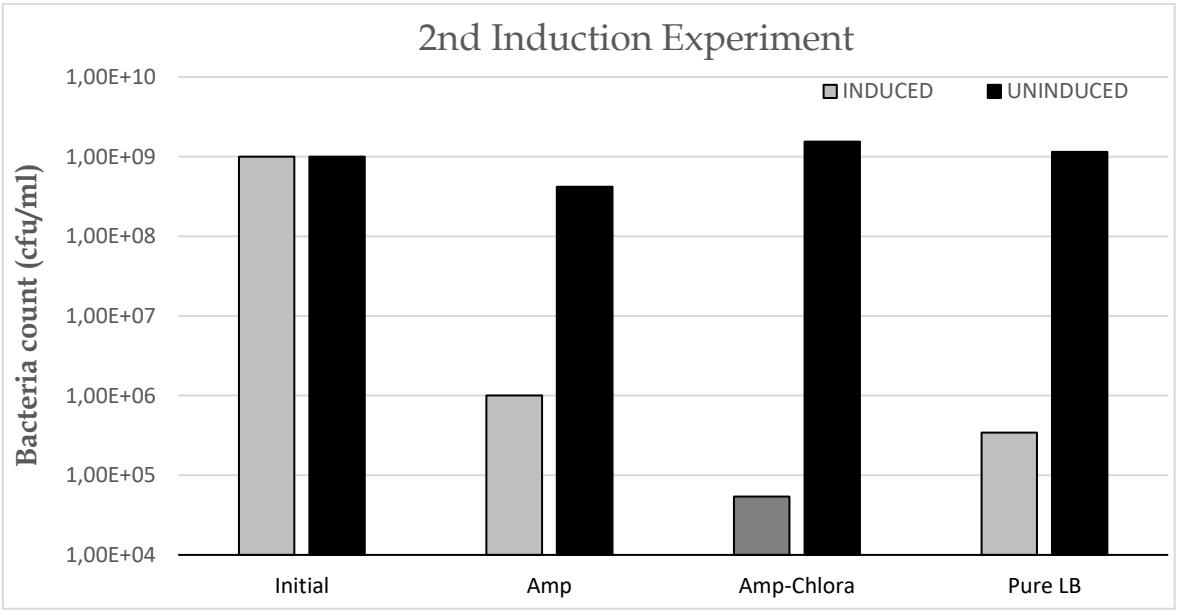

Figure 5. Calculated cfu counts for the second induction experiment. After the described induction of Cas9, induction cultures were plated on depicted antibiotic LB agar plates. Calculated cfu/ml on the logarithmic y-axis, which is cut at the 10^4 gridline. Antibiotic abbreviations on the x-axis: Ampicillin (Amp), Chloramphenicol (Chlora). Pure LB means a normal LB agar plate without any antibiotics. Induced samples in grey, uninduced in black.

Second induction experiment results were in line with the results from the first experiment, and now predicted results allowed better plating dilution selection for more reliable cfu counts. Growth is starkly hindered in the induced samples with ampicillin, but induction also heavily affects growth on plates without antibiotics (Figure 5).

Two more controlling induction experiments examined the fitness cost effect of tetracycline induction protocol on a strain without the pEC13 target plasmid (with the strain BL21 Gold (pLM2)(pCas9-oriT-IncFII), Figure 6) and without inducible Cas9 (with the strain BL21 Gold (pEC13), Figure 7).

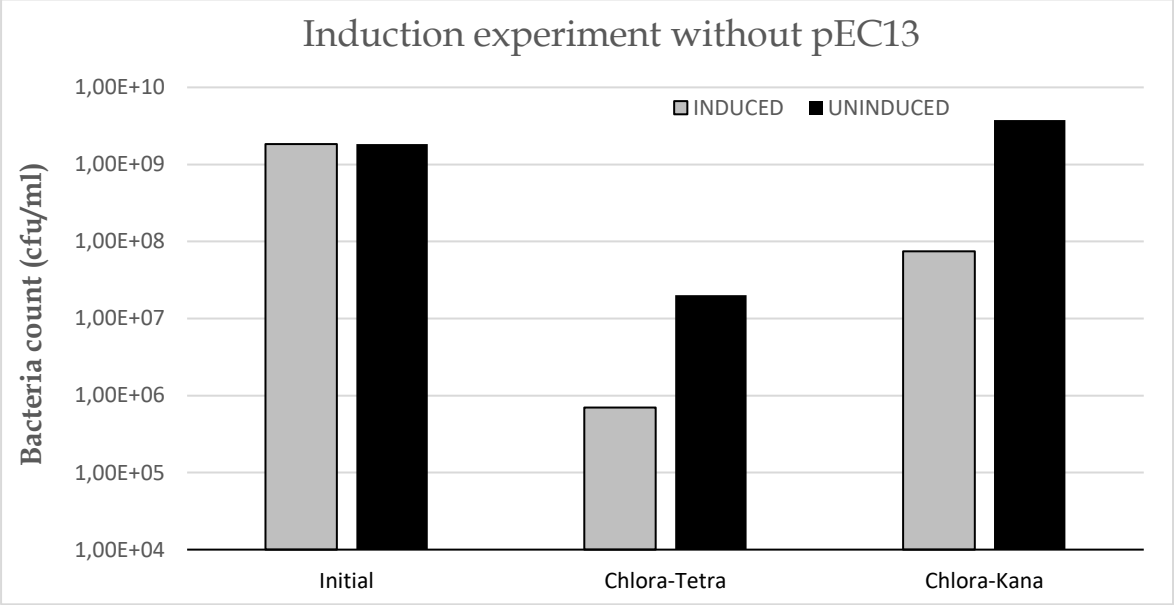

Figure 6. Calculated cfu counts for the induction experiment without the pEC13 target plasmid. After the induction, induction cultures were plated on depicted antibiotic LB agar plates. Calculated cfu/ml on the logarithmic y-axis, which is cut at the 10^4 gridline Antibiotic abbreviations on the x-axis: Chloramphenicol (Chlora), Tetracycline (Tetra), Kanamycin (Kana). Pure LB means a normal LB agar plate without any antibiotics. Induced samples in grey, uninduced in black.

The third induction experiment provided a valuable viewpoint on the Cas9 *tet* promoter mediated activation, as without the target pEC13 plasmid, *tet* activation had no other effect than Cas9 protein translation. Consequently, the only fitness costs in this experiment would have come from tetracycline resistance mechanisms (like before) and mechanisms relating to induced Cas9 translation, and the difference between induced and uninduced samples is relatively smaller (Figure 6) than in the first two experiments (Figure 4, Figure 5). Like in the first induction experiment, plates with tetracycline would have continued to induce Cas9, and that is reflected in the lowest cfu counts of this experiment.

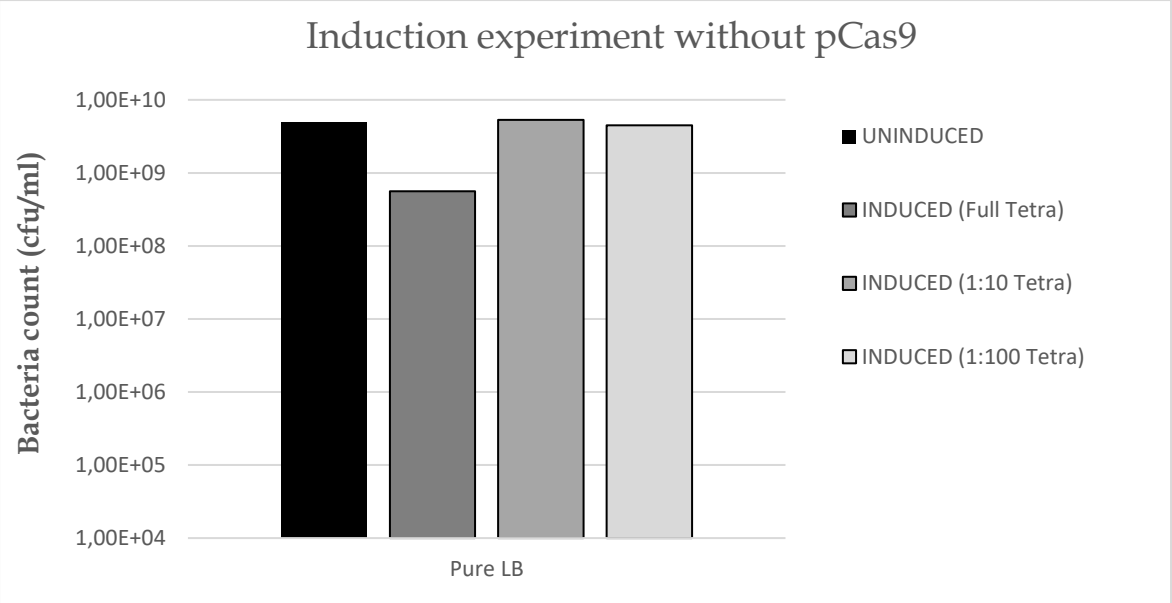

Figure 6. Calculated cfu counts for the induction experiment without the pCas9-oriT-IncFII plasmid. Calculated cfu/ml on the logarithmic y-axis, which is cut at the 10^4 gridline. Tetra is abbreviation for tetracycline. After the induction, induction cultures were plated on depicted pure LB agar plates (no antibiotics). Ampicillin-Tetracycline plates were uncountable, therefore not depicted. Uninduced sample in black and induced samples in grey: full tetracycline (20 µg/ml) in dark grey, 1:10 diluted tetracycline (2 µg/ml) in medium grey and 1:100 tetracycline (200 ng/ml) in light grey.

In the final induction experiment without pCas9, tetracycline dilution series was made for the induction, which allowed partial isolation of only tetracycline resistance-mediated effects on cells (Figure 7), although the lower dilutions (1:10 and 1:100) exhibited growth similar to the uninduced sample. Without Cas9 to be induced, tetracycline mediated its fitness cost effects through only through conventional Tet^R resistance mechanisms (see Discussion) and the difference between induced and uninduced samples was the smallest observed in the induction experiments.

4. DISCUSSION

Experimental work in this Master's Thesis was based on a hypothesis, that a conjugative CRISPR-Cas9 plasmid with *IncFII* crRNA could be cloned, conjugated to a new host and induced to cleave pEC13 sequence at the *IncFII* site. Nearly all the experiments were step-by-step testing of these different phases of the whole hypothesis. Although CRISPR-Cas9 is a very widely used molecular biology tool, literature based on this kind of specific idea was almost negligible, at least at the time. Nevertheless, a prior study by our group (Mattila *et al.* 2017) had shed a lot of light on the conjugation and properties of hospital pathogen ESBL plasmids (such as our pEC13 plasmid). After cloning the experimental pCas9-oriT-IncFII plasmid, the logical next step was conjugation testing.

In the first phase conjugation experiments, we set out to test the conjugation parameters to find optimal conditions for our two-plasmid conjugation (pCas9-oriT-IncFII mobilized via pLM2 conjugation machinery). First phase conjugation experiments showed that the pCas9-oriT-IncFII plasmid is indeed mobilizable through conjugation and is maintained in the transconjugant host, as proved by the antibiotic selection (Table 4, Table 5). Conjugation parameters clearly favoured conjugations with shaking incubator, compared with the stationary incubator, in our experimental transconjugants with the recipient HMS174 (pEC13), although the result was not clearly mirrored in the controlling recipient HMS174 strain (Table 4). This disparity could not be explained by differing starting recipient counts, as they were similar across all samples. On the other hand, shaking also produced higher total cell counts than a stationary incubator, and this generally improved growth can also partly explain the higher amount of transconjugants. Conjugation frequency varied widely, as a result of low transconjugant cfu counts. Additionally, donors were selected from the conjugation cultures using tetracycline, which would have induced the Cas9 *tet* promoter, resulting in a more fitness costs to plated donors. Still, tetracycline induction on a plate compared with a liquid culture yields different results, as discussed later.

First conjugation experiment transconjugants exhibited aberrant, small colonies, which indicated increased stress in the recipient cells with pLM2 and pCas9-oriT-IncFII (see below). Low colony counts with aberrant colonies complicated cfu counts, which can be partial explanation for slightly differing results between HMS174 (pEC13) and HMS174, as no duplicate plates were made. Aberrant colonies, with a low total colony count increased margins of error. Comprehensive conjugation frequency testing and optimization would have needed duplicate or triplicate plates for each dilution, preferably with clearly countable colony count in the optimal 25-250 range. With already high number of experimental parameters and plating dilutions, and without methods such as flow cytometry, conjugation frequency optimization was beyond the scope of this Master's Thesis.

One possible explanation for aberrant colonies was the heavy antibiotic load the bacteria were subjected on. First phase transconjugants were selected with kanamycin-rifampicin-chloramphenicol plates (Table 2), and although resistant, the transconjugants still would have had to utilize considerable resources to translate proteins relating to the resistance mechanisms, i.e. bearing the fitness cost. pCas9-oriT-IncFII had chloramphenicol (Cm^R) gene (Jiang *et al.* 2013) which codes for chloramphenicol acetyltransferase (CAT). CAT acetylates chloramphenicol molecules and prevents them from binding 50S ribosomal subunit. CAT activity allows protein translation to continue, but given the high number of ribosomes, some chloramphenicol molecules could still affect protein translation (Schwarz *et al.* 2004). Also, producing high quantities of proteins (like CAT), consumes cellular resources, thus affecting growth. Consequently, lower concentrations of chloramphenicol yielded higher cfu counts across all transconjugant categories in the next conjugation experiment (Table 5), but these could be just recipients without pCas9-oriT-IncFII, which survived on the diluted chloramphenicol plates. From the second conjugation experiment (Table 5), it was also apparent that a 1:1 volume ratio of donor to recipient was the more optimal scenario. Controlling culture with unmodified pCas9 plasmid yielded higher transconjugant cfu counts (roughly 10 times more bacterial cells) than the conjugation with the experimental

pCas9-oriT-IncFII plasmid in lower chloramphenicol concentrations ($\frac{1}{16}$ dilution and less). Unmodified pCas9 should not be able to conjugate, which would result to zero recipient transconjugants with resistance to all three antibiotics. As normal concentration chloramphenicol plates had no transconjugants for the unmodified pCas9, the higher cfu counts in the lower chloramphenicol concentrations were probably HMS174 (pEC13) recipients with conjugated pLM2, which survived the lesser chloramphenicol load (due to non-lethal mechanism of action). This observation partly led to minimum inhibitory concentration experiments described later. Some of the transconjugant colonies in the second conjugation experiment also exhibited aberrant morphology (Table 5 asterisks), which complicated the results, but this effect was not observed in all samples. A systematic or a plasmid based aberrant colony generation would have been easier to explain. The other explanation for these aberrant colonies, was that Cas9 translation is not exclusively driven by the *tet* promoter, and some kind of baselevel translational activity exists for the Cas9 gene, leading to crRNA-Cas9 mediated pEC13 cleaving and aberrant colonies. This possibility is further examined in the induction experiments discussion.

In the second phase conjugations, new strains allowed refined antibiotic selection screens. This time only two antibiotics, rifampicin and chloramphenicol, should screen for recipient transconjugants with pCas9-oriT-IncFII (Table 6). In the first and second conjugation experiments of the second phase, 1:1 donor to recipient ratio was shown to be the most optimal, although only slightly. Again, almost all the transconjugant colonies exhibited abnormalities, but the the conjugation frequency was best yet seen, which can partly be explained by the usage of only two antibiotics. The retainment of pEC13 in the transconjugants was not confirmed, without further antibiotic screens. Furthermore, second phase conjugation frequency calculations are approximations based on initial donor cfu count, as the conjugation cultures themselves were not screened for donors. This was an oversight, but conjugation frequency is still comparable within the second phase

experiments and can be used to approximate conjugation frequency also in relation to the first phase experiments as temporal lengths of conjugation remained low.

High antibiotic loads and aberrant colonies led to minimum inhibitory concentration growth curve experiments. These were done immediately after the first conjugation phase and the gained knowledge was used in the decision to change the conjugation bacterial strains from pair A to pair B in the second conjugation phase (Table 3). Like chloramphenicol, tetracycline binds to the ribosome (subunit 70S) blocking translation (Nguyen *et al.* 2014). This kind of protein synthesis inhibition does not necessary kill the cell but hinders its growth in the function of the antibiotic concentration. In our minimum inhibitory concentration experiments (Figure 2 and 3), protein synthesis inhibition is reflected on the results: chloramphenicol growth curves can be seen to follow a reasonably linear relationship in relation to the antibiotic concentration (Figure 3), but tetracycline exhibits this quality only at the lowest concentrations (Figure 2). Above the minimum inhibitory concentration (in 1:200 dilution), tetracycline growth curve exhibit deviancy: 1:300 dilution had two of its triplicates at the baseline, but one was able to grow (Figure 2), which, barring a mistake, implies some kind of mutation. On the other hand, chloramphenicol exhibited very regular relationship between the triplicates (not depicted). As both antibiotics have a similar mechanism of action, these results could not be explained by it. It is possible that the mechanisms of action for the antibiotic resistance can explain the difference (see below), and possibly some of the abnormal samples in tetracycline triplicates had acquired a spontaneous mutation enhancing growth.

Tetracycline resistance is mediated by the (tet^R) gene, which in *E. Coli* codes for a family of transmembrane efflux pumps. Like many similar active transporters, the pumps exchange a proton for a molecule of tetracycline against a concentration gradient. Although the resistance mechanism is considerably different compared with chloramphenicol resistance described earlier, both come with a fitness cost to the bacteria. As tetracycline would bind with a 70S subunit of the ribosome blocking translation, combatting this requires higher number of energetically more active

efflux pumps as tetracycline concentration rises (Nguyen *et al.* 2014), which was apparent from the induction experiments.

In the first and second induction experiments, results show that tetracycline induction lowers the cfu count by roughly three orders of magnitude in ampicillin-containing plates (Figure 4, Figure 5). This in line with the expectation, that induced cleavage by Cas9 ultimately renders pEC13 ESBL genes unusable, killing the hosts on ampicillin. Ampicillin-chloramphenicol plates (dark grey) depict the strain, which has to have pCas9-oriT-IncFII plasmids due to antibiotic selection, and the induced/uninduced difference is over three order of magnitude in the first experiment (Figure 4) and over four orders of magnitude in the second experiment (Figure 5). Additionally, differences between initial cell counts and uninduced sample counts can be partly explained by the 1:1 bacterial dilution in the induction protocol, but also by baselevel translational activity of Cas9, exclusive of *tet* promoter. But, like described, the tetracycline induction results in considerably higher Cas9 activation. In Figure 4, plates with ampicillin-tetracycline exhibit the lowest cell counts in both induced and uninduced samples, and this is likely due to induction on the plates, like described earlier for the unintentionally induced donor plates. Tetracycline efflux pump activity with Cas9 related translation hinder the growth even on a plate. Still, the ampicillin-tetracycline plates exhibit only a small (about one order of magnitude) difference in uninduced cfu counts compared with e.g. uninduced ampicillin-chloramphenicol cfu counts. On the other hand, difference is roughly three orders of magnitude between induced and uninduced ampicillin-chloramphenicol samples. This means that induction related effects are smaller in plates than in a liquid culture, but further experiments would have to be done to confirm this.

Although induced samples show multiple orders of magnitude lower cfu counts, there are also other mechanisms at play in induction: as described, tetracycline affects growth moderately linearly, as increased translation and activity of tetracycline efflux pumps in resistant strains comes with a fitness cost. Additionally, cleaved pEC13 plasmid may elicit other responses in the cell as well, as pEC13

contained *RelE* toxin-antitoxin system (See Table 3: GenBank: KU932024.1). Linearized pEC13 could deregulate the plasmidial toxin-antitoxin system, leading to antitoxin degradation and eventually toxin-driven cell death. Toxin-antitoxin or analogous system could explain the difference the large difference between induced and uninduced samples in pure LB plates (Figure 4, Figure 5). On pure LB plates, the only additional fitness cost to the bacteria from induction should be the tetracycline efflux pumps and Cas9 related translation, if pEC13 linearization would not cause additional growth impairment. This kind of scenario was examined in the third induction experiment, without the target pEC13 plasmid (Figure 6). From Figure 6, it is apparent that tetracycline induction on chloramphenicol-kanamycin plates exhibits additional fitness cost, probably from tetracycline efflux pumps and Cas9 translation, as no target pEC13 plasmid is present. This results in difference of a bit over one order of magnitude between the induced and uninduced samples. In the last induction experiment without the pCas9, the difference between (full concentration tetracycline) induced and uninduced sample is again a bit over one order of magnitude (Figure 7). This in in the same effect range like the observed result from Figure 6. Without inducible Cas9, the only fitness cost from induction should come from the tetracycline efflux pumps. Lower dilution induction samples of tetracycline in Figure 7, exhibit similar growth to the uninduced sample, but Cas9 induction was not tested with these lower tetracycline induction dilutions. If the effect of tetracycline efflux pumps and merely Cas9 translation is roughly one order of magnitude (Figure 6, Figure 7), then pEC13 cleavage should account for the remaining two to three orders magnitude difference between induced and uninduced samples in Figures 4 and 5. Even with the toxin-antitoxic system present, with cleaved pEC13 the bacteria would primarily die to ampicillin, as it is lethal to bacteria without ESBL plasmids (Li *et al.* 2019), confirming successful pCas9 induction and subsequent pEC13 cleavage. Others have used a similar pCas9 system, but without conjugation, and reported 99% of cells dead with CRISPR-Cas9-mediated cleavage of the ESBL plasmid on ampicillin plates (Kim *et al.* 2016)

4.1 Conclusions

From the very beginning, we set out to test different phases of the experimental hypothesis and ultimately come to a successful proof-of-principle concept with inducible pCas9-oriT-IncFII plasmid, that was mobilizable via pLM2's conjugation channel. Plasmid was successfully cloned, and it was definitely possible to conjugate it to a new host, although conjugation optimization was not complete. Plasmid's induction lowered the cfu count of ESBL plasmid-hosting bacteria by roughly three orders of magnitude on β-lactam plates. Ultimately, successful proof-of-principle was reached.

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APPENDIX 1. Experimental oligonucleotide sequences.

Sequence name	Sequence (5′-3′)	Oligonucleotide length
Forward OriT813	TATATAGTCGACCGACAGGCTCATGCC	27
Reverse OriT813	ATATATGTCGACAGCCTTGCCCCCCT	26
Forward IncFII	AAACCGAAAAACTGATGGAAAAGGG	25
Reverse IncFII	AAAACCCTTTTCCATCAGTTTTTCG	25
Reverse crRNA PCR probe	TCACACTACTCTTCTTTTGCCTATTATAACAT	32

APPENDIX 2. PCR programs.

oriT PCR (two-step) program:

7 min	98 °C	Initial denaturation	
10 s	98 °C	Denaturation	34x
30 s	72°C	Annealing & Extension	
7 min	72 °C	Final Extension	
hold	4 °C		

IncFII PCR (three-step) program:

7 min	98 °C	Initial denaturation	
10 s	98°C	Denaturation	
15 s	68°C	Annealing	34x
20 s	72°C	Extension	
7 min	72°C	Final Extension	
hold	4 °C		