Development of methods for genetic manipulation of bacteria from order Bacillales

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GRADUATE THESIS

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Development of methods for genetic manipulation of bacteria from order *Bacillales*

Experimental work for this Graduate thesis was done at the Technical University of Denmark (DTU), DTU Bioengineering, Section for Microbial and Chemical Ecology, Bacterial Interactions and Evolution group, Denmark. The thesis was made under the guidance of professor Ákos T. Kovács and with the help of PhD student Yicen Lin.

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Development of methods for genetic manipulation of bacteria from order *Bacillales*

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Abstract: Strains from order *Bacillales* have numerous industrial applications but due to the complexity and non-efficient transformation protocols have not reached their full implementation potential. This Master Thesis was focused on the transformation of selected strains from order *Bacillales*: *Bacillus thuringiensis 407 cry-*, *Paenibacillus sp., Bacillus simplex, Lysinibacillus fusiformis M5,* and *Bacillus velezensis FZB42.* In order to perform and follow their transformation, two vectors labelled through introduction of a green fluorescent protein gene were constructed by using Gibson assembly. Within transformation protocols special attention was paid to electroporation, suitable for the transformation of *B. thuringiensis 407 cry-*, and conjugation, optimal for the rest of the tested strains. Further, stability of the two constructed plasmids and two other, previously prepared plasmids, used in the transformation of *B. thuringiensis 407 cry-*, was tested by using fluorescence and resistance to antibiotics (kanamycin and chloramphenicol). In addition, plasmid gene expression was followed during growth of *B. thuringiensis 407 cry-* by stereomicroscopy, root colonization assay, and confocal laser scanning microscopy. Based on obtained results it can be concluded that successfully transformed *B. thuringiensis 407 cry-* is good candidate for plant-growth-promoting bacterium.

Keywords: *Bacillales, Bacillus thuringiensis,* Gibson assembly, transformation, plant-growth-promoting bacterium
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Razvoj metoda za genetičke manipulacije bakterija iz reda *Bacillales*

Tomislav Vološen 1170/BPI

Sažetak: Vrste iz roda *Bacillales* imaju široku primjenu u industrijskoj proizvodnji, ali zbog kompleksnosti i neučinkovitosti protokola za njihovu transformaciju još uvijek nisu dosegli svoj puni industrijski potencijal. Ovaj je diplomski rad bio usmjeren na transformaciju odabranih vrsta iz reda *Bacillales*: *Bacillus thuringiensis 407 cry-, Paenibacillus sp., Bacillus simplex, Lysinibacillus fusiformis M5* i *Bacillus velezensis FZB42.* Primjenom Gibsonove metode konstruirana su dva vektora, u koje je kao marker uveden gen za zeleni fluorescirajući protein. Ova su dva vektora korištena za optimiranje protokola za transformaciju pobrojenih vrsta, u okviru kojeg je posebna pozornost posvećena postupku elektroporacije, koji se pokazao učinkovitim za transformaciju *B. thuringiensis 407 cry-*, i konjugacije, koji je bio optimalan za preostale odabrane vrste iz ovog reda. Nadalje, stabilnost dvaju konstruiranih plazmida kao i dva prethodno pripremljena plazmida, koji su se koristili za transformaciju *B. thuringiensis 407 cry-*, testirana je uz primjenu metode za određivanje fluorescencije i rezistencije na antibiotike (kanamicin i kloramfenikol). Dodatno, praćena je ekspresija gena plazmida tijekom rasta *B. thuringiensis 407 cry-* metodama stereomikroskopije, kolonizacije korijena biljke i konfokalnom laserskom skenirajućom mikroskopijom. Na temelju dobivenih rezultata može se zaključiti da je uspješno transformirani *B. thuringiensis 407 cry-* dobar kandidat za grupu bakterija koje pospješuju rast biljaka.

Ključne riječi: *Bacillales, Bacillus thuringiensis,* Gibsonova metoda, transformacija, bakterije koje pospješuju rast biljaka

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1. INTRODUCTION

Order *Bacillales* present a huge group of bacterial strains that are quite special due to their metabolic possibilities and offer huge potential for exploitation of their characteristics in biotechnology. Some of the strains from this order are pathogenic (Levinson, 2016) but, on the other hand, have industrial potential which makes them hot area for research. This Thesis was mainly concentrated on bacteria from *Bacillus cereus* Group, especially *Bacillus thuringiensis.* The main goal of this Thesis was to develop genetic tool which can be used to better understand physiology and metabolism of these bacteria. The Thesis has been separated in few small projects.

First task of this Thesis was to construct conjugative plasmid that is labelled through introduction of a green fluorescent protein (GFP) gene, so that fluorescence of co-expressed genes (the gene of interest and GFP gene) serves as an indicator of successful transformation and expression. Gibson assembly was planned to be used for cloning of two fragments (conjugative vector and fragment containing GFP). Then, successful cloning into receiving plasmid can be tested with several methods (colony PCR, plasmid digestion with restriction enzymes, and GFP fluorescence).

Second task was construction of plasmid that has active CRISPR-Cas9 system for *B. thuringiensis* genome editing, by the assembly of four different fragments. This plasmid should have GFP gene in between two homologous regions of *B. thuringiensis* genome and active CRISPR-Cas9 system that introduce double-stranded break in the *B. thuringiensis* genome and subsequently repair double-strand using homologous recombination. To reach this particular goal, plasmid has to have single-guided RNA (sgRNA) which should be introduced in the plasmid by Golden-gate cloning and Gibson assembly of four fragments. We had in mind that there might be difficulties due to efficacy of the assembly which is noticeably reduced when higher number of fragments have been used (Gibson et al., 2009).

Third, it was important to create an efficient transformation protocol for strains from order *Bacillales*. The transformation protocol should include electroporation or conjugation.

In the end, plasmid gene expression should be followed during growth at least one of the successfully transformed strain by different methods and define if any of selected strain from the order *Bacillales* is suitable as plant-growth-promoting bacterium.

2. THEORETHICAL PART

2.1. A BRIEF OVERVIEW OF CLASS Bacilli

Bacterial taxonomic class Bacilli (Bergey et al., 2009) is a huge group of bacterial strains where almost all bacteria are gram-positive. Bacilli class has two taxonomic orders *Bacillales* and *Lactobacillales.* In this thesis bacteria from order *Bacillales* have been employed, specifically from genus *Bacillus* and two newer genus *Paenibacillus, and Lysinibacillus (*Ash, et al., 1993). In appendices is shown the phylogenetic tree of *Bacillus* genus.

2.1.1 *Bacillus thuringiensis*

Majority of the experiments in this thesis have been done with *B. thuringiensis* gram-positive, aerobic, spore-forming bacteria. It belongs in the *Bacillus cereus* group which includes several *Bacillus* species with closely related phylogeny (closest relatives *B. anthracis,* and *B. cereus)* (Ehling-Schulz et al., 2019). Even though *B. anthracis* (anthrax causative agent (Turnbull, 1996)) and *B. cereus* (food-poisoning syndromes (Drobniewski, 1993)) are pathogenic bacteria, and difference from *B. thuringiensis* is mainly in plasmid DNA (Økstad and Kolstø, 2010), *B. thuringiensis* is not human pathogen.

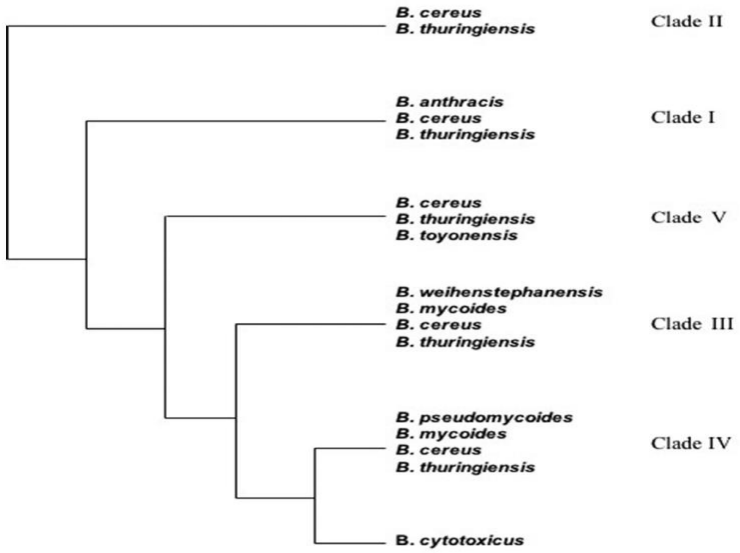


Figure 1. The five major phylogenetic clades of the *B. cereus* group. Clade I- *B. anthracis* clade, Clade II- *B. cereus/B. thuringiensis* clade, Clade III- *B. weihenstephanensis* clade, Clade

IV- strains belonging to various *B. cereus* group species. Kindly provided by Ehling-Schulz (Ehling-Schulz et al., 2019).

Bacillus thuringiensis is well known for its insecticidal activity. Insecticidal activity is mainly present due to production of crystalized δ-endotoxin (also termed Cry proteins). The toxin is activated in the insect's gut before binding to epithelial cell-specific receptors, causing cell lysis and death (Fedhila et al. 2002). Due to the production of insecticide, bacteria are commercial biopesticide having worldwide application in forestry and agriculture (Broderick et al., 2006). The popularity of *B. thuringiensis* and toxin is due to fact that toxin is biologically degradable, selectively active on pests, and less likely to cause resistance in comparison to chemical pesticides (Alvarez et al., 2009). The strain used in this thesis is *Bacillus thuringiensis 407 cry-* which means that it is not producing δ-endotoxin.

Furthermore, *B. thuringiensis* is soil, biofilm-forming bacteria (Kuroki et al., 2009). In Figure 2., it is possible to see *Bacillus subtilis* biofilm composition which is similar to *B. thuringiensis* composition. As well, *B. thuringiensis* should colonise roots and other materials by producing biofilm on them. Additionally, *Bacillus* root biofilms can promote and protect plant growth (Vidal-Quist et al., 2013).

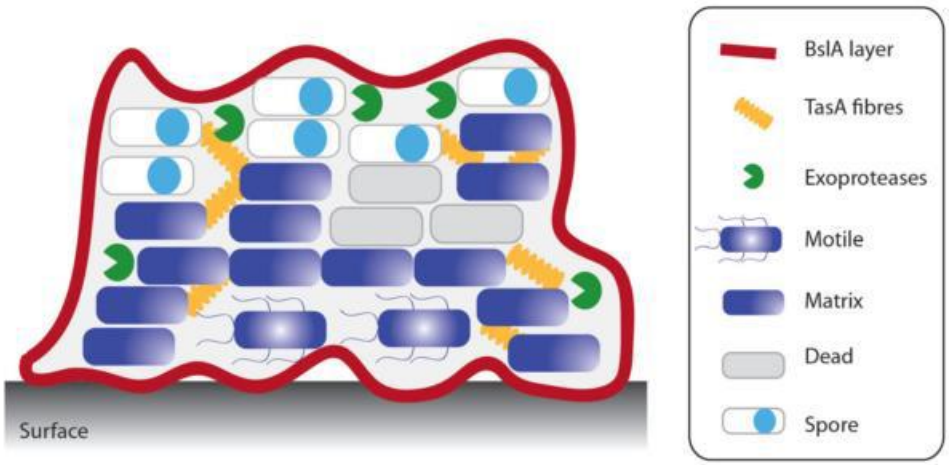


Figure 2. Composition of *B. subtilis* biofilm. The BslA protein coats *B. subtilis* biofilm and the TasA amyloid fibres give structure to the biofilm. Kindly provided by Hobley (Hobley et al., 2015).

2.1.2. *Bacillus simplex*

Bacillus simplex is Gram-positive, aerobic, and spore-forming bacteria. As well, *B. simplex* is soil bacteria, root colonizer that has a positive effect on plant growth. It is often called plant-growth-promoting bacteria (PGPB), which is used to describe microbes that

colonize and has a positive impact on surface of stems, leaves, seeds, and roots. Additionally, certain *B. simplex* strains promote growth of kiwifruit, Arabidopsis, tomato, and wheat (Schwartz et al., 2013). As well it was observed that *B. simplex* has antifungal activities on *Heterobasidion annosum*, *Armillaria mellea*, *and Pythium aphanidermatum* (Miao et al., 2018). Lastly, after genome sequencing of *B. simplex* strain WY10 it was shown that this was first strain to be functionally characterized as naturally competent and the first Gram-positive bacterium to demonstrate compatibility with the *Escherichia coli* plasmid p$\pi\gamma$ (R6K origin) (Keen et al., 2017).

2.1.3. *Paenibacillus*

Paenibacillus genus, previously classified within the genus *Bacillus*, represent facultative anaerobic, endospore-forming, periflagellated heterotrophic and low G+C Gram-positive bacteria. *Paenibacillus* strains have been isolated from a variety of environments, and produce a variety of secondary metabolites, such as enzymes, exopolysaccharides, biosurfactants, and antibacterial and antifungal metabolites (Bach et al., 2016). Also, *Paenibacillus* is good root colonizer that can survive different types of soil, and they play an important role in agriculture (Brito et al., 2016).

2.1.4. *Bacillus velezensis*

Bacillus velezensis is Gram-positive, plant-growth-promoting bacteria (PGPB). It has phytostimulatory and biocontrol action. In agriculture *Bacillus velezensis* FZB42 is successfully used as biofertilizer and biocontrol bacteria. Previously it was named *Bacillus amyloliquefaciens subs. plantarum. B. velezensis* has shown good abilities to produce enzymes like amylases, glucanases and proteases (Fan et al., 2018).

2.1.5. *Lysinibacillus fusiformis*

Lysinibacillus fusiformis is Gram-positive, rod-shaped, obligate aerobe, endospore-forming bacteria. Natural habitat is various, but mostly it has been isolated from farming soil and factory wastewater (Bergey et al., 2009).

Reason why for strains, like *Lysinibacillus fusiformis, B. simplex,* and some *Paenibacillus sp.,* there is no enough of available data is that it is really hard to work with them,

and even harder to transform (Barchewitz and Kovács., 2016) and that is why there is requirement to develop appropriate vectors and efficient transformation protocols.

2.2. MOLECULAR CLONING METHODS

Molecular cloning is a set of methods that allows us to assemble recombinant DNA. Cloning provides us with opportunity to better understand genome organization and gene expression, production of recombinant proteins, gene therapy, and nowadays it is crucial tool in every microbiology lab.

2.2.1. Theory behind Golden-gate cloning

Golden-gate cloning is a method that allows a simultaneous and directs assembly of multiple DNA fragments. The simplicity of this method provides us with very efficient method of cloning. Principe of method lies in using restriction enzyme Type IIs that cut DNA outside of their recognition sites. Type IIs restriction enzymes are BsaI, BsmBI, and BbsI (Ott et al., 2016).

Due to the fact that restriction enzymes cut DNA outside of recognition sites, it creates different overhangs. These overhangs are now fusion sites that are practicable for assembly of multiple fragments. In practice that means that in reaction mix we can put all fragments still in plasmids, destination vector, Type IIs restriction enzyme, and T4 DNA ligase and we will still have extremely high efficiency (Weber et al., 2011). In Figure 3. it is possible to see method of golden-gate cloning.

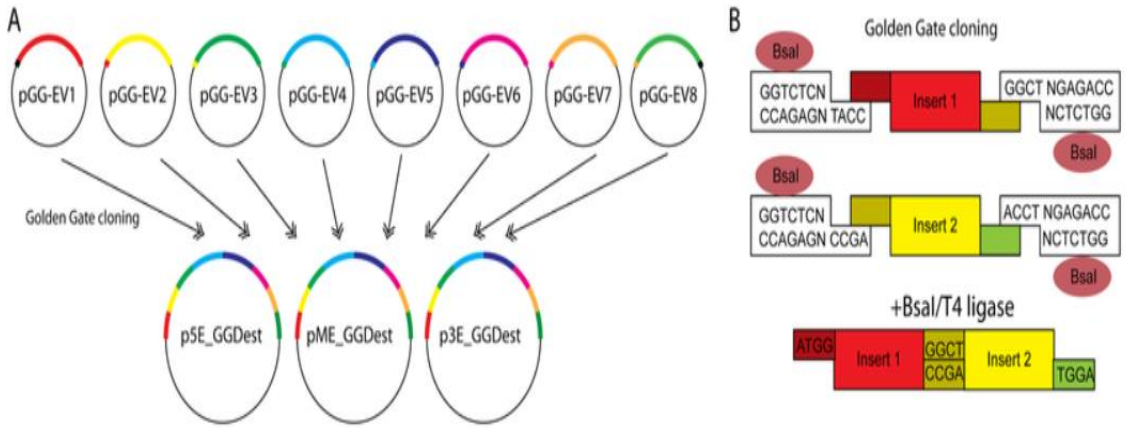


Figure 3. Golden-gate cloning method. Kindly provided by Kirchamier (Kirchmaier et al., 2013).

2.2.2. Theory behind Gibson assembly

Gibson assembly is a very useful and resourceful method that can in single reaction assemble multiple fragments with different length, and amount of overlap (Gibson et al., 2010). Gibson reaction master mix contains T5 exonuclease, Phusion DNA polymerase, and *Taq* DNA ligase. It can be built by ourselves or bought by New England BioLabs.

It is a really elegant, and low-time consuming method that doesn't require any cleanup step (Deyling et al., 2018), but in order to achieve DNA annealing, fragments should have 20-150bp overlaps. In practice T5 exonuclease clean 5' end of the fragments creating single-stranded DNA that is designed to anneal. After annealing DNA polymerase fills the gaps, and at the end, *Taq* ligase seals the nicks (Røkke et al, 2013). In Figure 4. it is possible to see overview of the Gibson assembly cloning method.

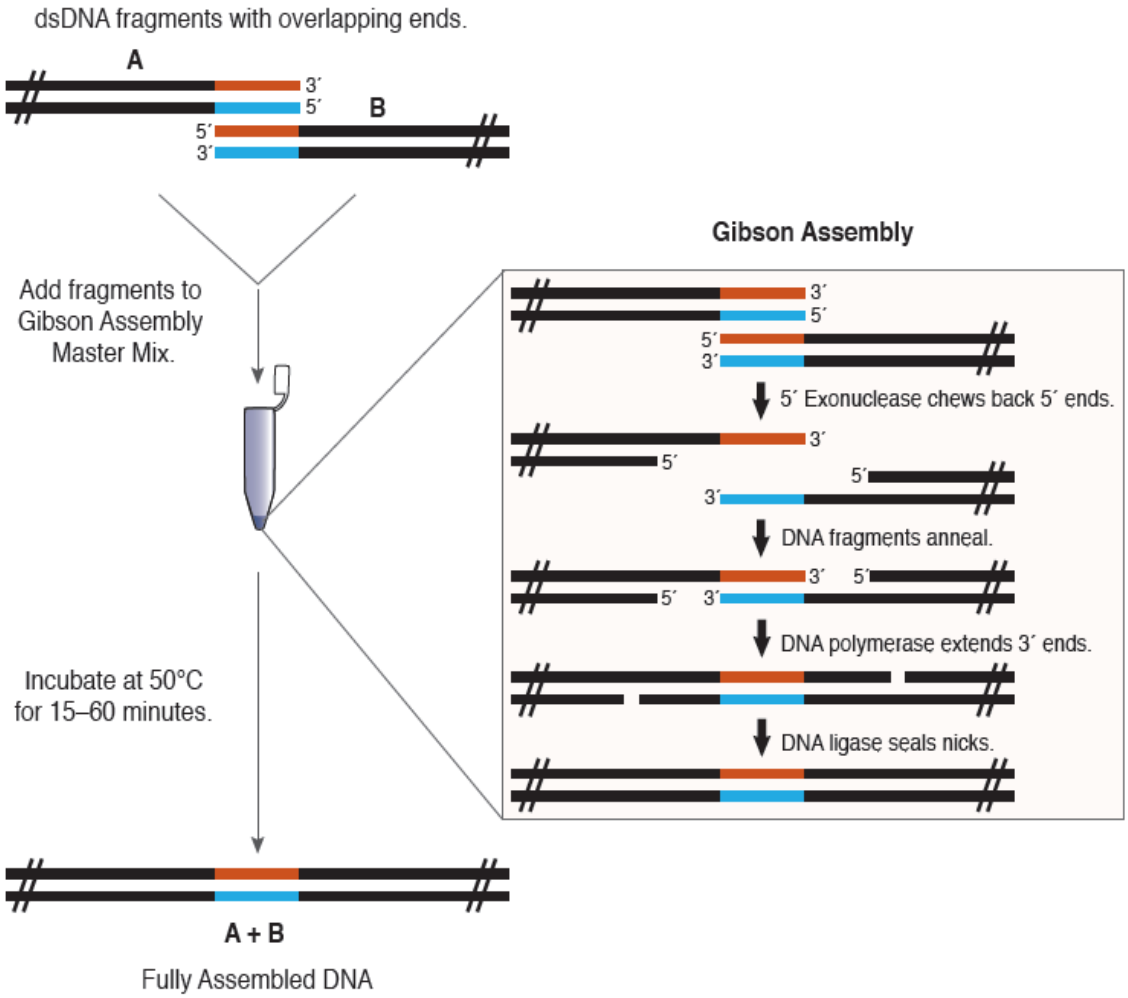


Figure 4. Overview of the Gibson assembly method available in New England BioLabs protocol for Gibson assembly

2.3. TRANFORMATION TECHNIQUES

A transformation is a useful tool that provides us with ability to introduce foreign DNA into cell and with that cause modification of transformed cell. In this research, used transformation techniques are electroporation and conjugation.

2.3.1. Theory behind Electroporation

Transformation by electroporation is a membrane phenomenon which involves fundamental behaviour of the cell. The basic principle is that presence of electric field interacts with lipids from cell membrane causing pores threw which DNA can enter the cell (Weaver and Chizmadzhev, 1996). The method is shown in Figure 5.

Electroporation was firstly mentioned in 1982 by Neumann who employed electroporation to transfer mouse lyoma cells and has been used for in vivo gene transfer since 1991. Mayor roll in electroporation system play electrode. Until today there has been developed multiple types of electrodes, but the best working one is multielectrode array which provides us with higher transfection rate and less damage. Even though this method is very popular and stable, transformation efficiency is not stable probably due to the fact that cell viability is still low after electroporation (Du et al, 2018).

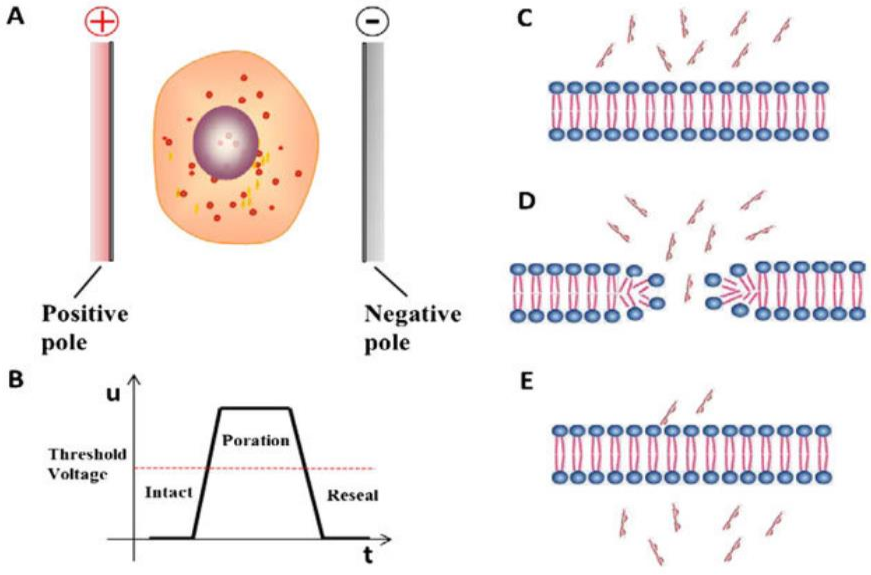


Figure 5. The principle of electroporation. When a live cell is exposed to the electric field (A.C), due to the high voltage, pore is formed (D) allowing DNA to enter the cell. In the end, cell membrane repair to the original state with exogenous DNA inside. Kindly provided by Du (Du et al., 2018).

2.3.2. Theory behind Conjugation

Conjugation is a very interesting method of transformation. It is natural process that happens in nature but can be controlled in lab conditions. Conjugation of bacteria is a process in which plasmid is transferred by themselves from one cell to another through conjugation tube. The cell that transfer plasmid is called donor, and the one that receives is recipient (Ou and Anderson, 1970). The figure of sex pili or conjugation tube is shown in Figure 6.

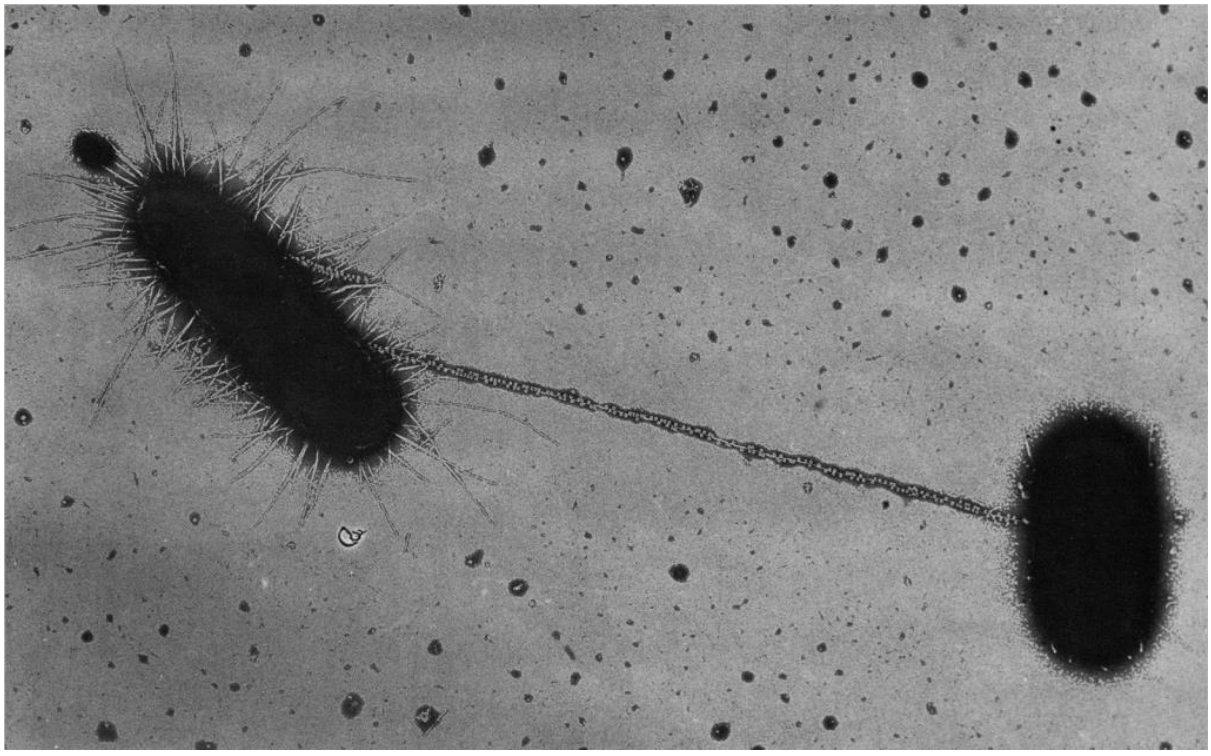


Figure 6. Scanning electron micrograph of conjugation by conjugation tube (pili) in bacteria. Kindly provided by Leitenberg (Leitenberg, 1967).

Conjugative plasmids, also known as F-factor or F-plasmid, need to have Tra gene (transacting gene) and OriT site (origin of transfer; part of plasmid DNA from which transfer is initiated). Tra gene is responsible for preparing plasmid for transfer by providing them with enzymes and primase (primase is transferred to recipient cell to complete the replication of another strand of plasmid DNA) (Derbyshire and Gray, 2014). Conjugation described by steps is shown in Figure 7.

In the novel research by Feuillie, it was proven that Gram-positive bacteria have different way of matting, not by using conjugation pili like Gram-negative bacteria. In Gram-positive bacteria there are some encoded cell surface proteins that are believed to be responsible for the aggregation of mating partners (Feuillie et al., 2018).

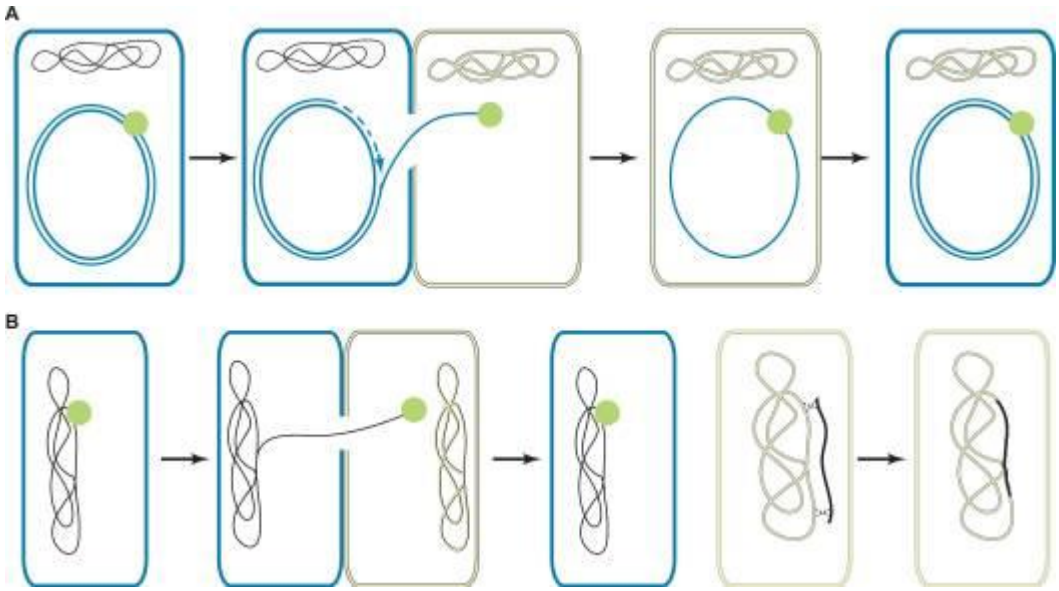


Figure 7. Steps and mechanism of transfer of plasmid from donor to recipient cell. (A) transfer that occurs in F-strains (just transfer of plasmid DNA). (B) transfer that occurs in Hfr-strains (conjugative plasmid is integrated into the bacterial genome). Kindly provided by Derbyshire and Gray (Derbyshire and Gray, 2014).

3. EXPERIMENTAL PART

3.1. MATERIALS

3.1.1. Chemicals

The chemicals and antibiotics used in this thesis were obtained from companies Carl-Roth GmbH &Co. Kg (Germany), and Sigma-Aldrich Corporation (St. Louis, MO, USA). Enzymes and related solutions were obtained from Thermo Fisher Scientific (Waltham, MA, USA), and New England Biolabs (Ipswich, MA, USA).

3.1.2. Media and cultivation of bacteria

Bacilli strains, used in this thesis, were grown on 30 ℃, 220 r.p.m. or in the incubator at 30 ℃, but the different medium has been used. Cultivation of *Escherichia coli* was carried out at 37 ℃, 220 r.p.m. or an incubator at 37 ℃ in the complex LB (Lennox) media (Carl Roth GmbH). For all different media the standard agar concertation used was 1.5 %w/v. List of buffers and media can be found in Table 1. and Table 2. All buffers and media were fixed in pH rage 7.0-7.2, respectively.

Table 1. Cultivation media used for Bacilli and *E. coli.* For each medium the used components and their concertation respectively used quantity is shown.

LB Lennox		Nutrient broth	
Component	Concertation	Component	Concertation
Tryptone	10 g L^{-1}	Beef extract	3 g L^{-1}
Yeast extract	5 g L^{-1}	Peptone	5 g L^{-1}
Sodium chloride	5 g L^{-1}	Sodium chloride	5 g L^{-1}
		Glucose	2 g L^{-1}
BHI (Brain-Heart-Infusion broth)		King's broth	
Component	Concertation	Component	Concertation
Pig brain infusion	7.5 g L^{-1}	Peptone	20 g L^{-1}
Pig heart infusion	10 g L^{-1}	Glycerol (100%)	10 mL
Peptone	10 g L^{-1}	Di-Potassium Hydrogen Phosphate	1.5 g L^{-1}
Glucose	2 g L^{-1}	Magnesium Sulphate Heptahydrate	1.5 g L^{-1}
Sodium chloride	5 g L^{-1}		
Disodium phosphate	2.5 g L^{-1}		

Table 2. Buffers used in this thesis. For each buffer used components and their concertation respectively used quantity is shown.

Electroporation buffer	
Component	Concentration
Sucrose	272.0 mmol L^{-1}
Magnesium chloride	0.5 mmol L^{-1}
di-Potassium Hydrogen Phosphate	0.5 mmol L^{-1}
Potassium dihydrogen Phosphate	0.5 mmol L^{-1}

RF1 buffer	
Component	Concentration
Rubidium chloride	12 g L^{-1}
Potassium chloride	4.9 g L^{-1}
Calcium chloride	1.5 g L^{-1}
Glycerol	150.0 g L^{-1}

RF2 buffer	
Component	Concentration
0.5 M MOPS	6 mL
Rubidium chloride	1.2 g L^{-1}
Calcium chloride	11.0 g L^{-1}
Glycerol	150 g L^{-1}

For *Arabidopsis thaliana* growth MS media (Murashige and Skoog basal salt mixture, Sigma) has been used and its components and concertation are shown in Table 3.

Table 3. Components and concentration of MS (Murashige and Skoog media)

Component	Concentration (mg L^{-1})
Ammonium nitrate	1650.0
Boric acid	6.2
Calcium chloride anhydrous	332.2
Cobalt chloride X $6H_2O$	0.025
Cupric sulphate X $5H_2O$	0.025
Na_2-EDTA	37.26
Ferrous sulphate X $7H_2O$	27.8
Magnesium sulphate	180.7
Manganese sulphate X H_2O	16.9
Molybdic acid (sodium salt) X $2H_2O$	0.25
Potassium iodide	0.83

Potassium nitrate	1900.0
Potassium phosphate monobasic	170.0
Zinc sulphate X $7H_2O$	8.6
Glycine (free base)	2.0
Myo-inositol	100.0
Nicotinic acid (free acid)	0.5
Pyridoxine X HCl	0.5
Thiamine X HCl	0.1

3.1.3. Bacterial strains and plasmids

All strains and plasmids used in this thesis are listed in Table 4. *E. coli* JM109 has been used for chemical transformation after cloning and as host of plasmid DNA. *E. coli* S17 has been plasmid donor in transformation by conjugation (Toymentseva and Altenbuchner, 2018). All Bacilli strains have been used for transformation by electroporation and conjugation. As well as *Bacillus thuringiensis* strains have been used in root colonization assay and plasmid stability assay. The plant used for root colonization was *Arabidopsis thaliana.*

Table 4. Strains and plasmids used in this thesis**. ***EE refers to evolved strains from 407 cry- strain. Plasmid's DNA sequence and a short description are shown in appendices.

Strains or plasmids	Description	Origin or reference
Strains		
Bacillus thuringiensis strains:		
407 cry-	Wild type strain	Lereclus et al. 1989
EE-A*	Unidentified	Not published
EE-C*	Unidentified	Not published
EE-D*	Unidentified	Not published
EE-E*	Unidentified	Not published
EE-F*	Unidentified	Not published
Paenibacillus sp. 037	*Paenibacillus* closely related to *P. barcinonensis*	Not published
Paenibacillus sp. 040	No whole-genome sequencing has been done	Not published
Bacillus simplex 050	closely relative to *B. pocheonensis*	Not published

Lysinibacillus fusiformis M5	*L. fusiformis M5* wild type isolate	Gallegos-Monterosa et al. 2016
Bacillus velezensis FZB42	Unidentified	Not published
Escherichia coli JM109	*endA1, glnV44, thi-1, relA1, gyrA96, recA1, mcrB*$^{+}$, Δ(*lac-proAB*), *e14-*, [F' *traD36 proAB*$^{+}$ *lacI*^{q} *lacZΔM15*], *hsdR17*($r_K^- m_K^+$)	Yanisch-Perron *et al.*, 1985
Escherichia coli S17-1	*recA*, *pro* (RP4-2Tet::Mu, Kan::Tn7)	Simon et al., 1983
Arabidopsis thaliana	//////////////////////////////	Clough and Bent, 1998
Plasmids		
pJOE9734.1	Kan^{R}, P_{manPA}-*cas9*, P_{vanP*}, *lacPOZ'*-gRNA, *oop ter*, T7P, *lacZα-traJ/oriT-lacZ*, *repE194*^{ts}, pUC*ori*	Toymentseva and Altenbuchner, 2018
pJOE9732.4	Kan^{R}, *xylR*-P_{xylA}-*cas9*, *lacZα*-gRNA, *repE194*^{ts}, pUC*ori*	Toymentseva and Altenbuchner, 2018
phyGFP	Plasmid to amplify hyGFP	Van Gestel et al., 2014
pNW33N	Chloramphenicol resistance; vector for fluorescent reporters	Bacillus Genetic Stock Center
pTB603	pNW33n-hyGFP	Barchewitz and Kovács., 2016
pTB604	pNW33n-hymKATE	Barchewitz and Kovács., 2016
pDTUB184	pJOE9734.1- Pman-hyGFP	This work
pDTUB185	pJOE9734.1-hyGFP	This work

All primers used in this thesis are listed in Table 5.

Table 5. List of all primers.

Primmer	Sequence (5'→3')
oTV01	cgtatttaactagtaccctgcttcgggg
oTV02	attatgccacacctttgtctcaactgtataccgaaatc
oTV03	gaccccgaagcagggtactagttaaatacgcttcacagtttct
oTV04	tatacagttgagacaaaggtgtggcataatgtgt
oTV05	attatgccacaccttgtcatgacattggtgtacag
oTV06	caccaatgtcatgacaaggtgtggcataatgt
oTV07	CCGGATGTGAGCGCCGGTgcttcggggtcattatagcgatttttcg
oTV08	CATTATGCCACACCTTGttgtctcaactgtataccgaaatcagctcattaaaat

oTV09	tataatgaccccgaagcACCGGCGCTCACATCCG
oTV10	ggtatacagttgagacaaCAAGGTGTGGCATAATGTGTGT
oTV11	CATTATGCCACACCTTGgttccactgagcgtcagaccc
oTV12	tctgacgctcagtggaacCAAGGTGTGGCATAATGTGTGTAATTG
oTV13	agtctagtgccccaagttccc
oTV14	gtgcgtaacttctcatctcatctacgt
oTV15	tacgaaaggtaaatgaacatttcg
oTV16	aaaccgaaatgttcatttaccttt
oTV17	ggccaacgaggcccgggccagcgaagtctagtgcc
oTV18	CGCTCACAATTACACACAacgcatcccccaagttttttaattg
oTV19	aaacttgggggatgcgtTGTGTGTAATTGTGAGCGGATAACAATTAAGC
oTV20	ctttggaaaacgattctaAAGCCTTGCATATCCTGCAAGGT
oTV21	CAGGATATGCAAGGCTTtagaatcgttttccaaagcggatgtatcg
oTV22	ccccgatttagagcttgagagtgcgtaacttctcatctcatct
oTV23	tgagaagttacgcactctcaagctctaaatcgggggct
oTV24	ggcactagacttcgctggcccgggcctcgtt
oTV25	tcgacggccaacgaggcttccgccagcgaagtctagt
oTV26	gagtcagctaggaggtgaagagtgcgtaacttctcatctcatct
oTV27	gagaagttacgcactcttcacctcctagctgactcaaatcaat
oTV28	tagacttcgctggcggaagcctcgttggccgtcgac
oTV29	tcgctataatgaccccgtgtgaacgacgtttccgccag
oTV30	gatacatccgctttggaacgtatttttcccacacgcatcc
oTV31	cgtgtgggaaaaatacgttccaaagcggatgtatcggtgaaa
oTV32	tcagctaggaggtgactgaaacaggctaggaagaagactttct
oTV33	ttcttcctagcctgtttcagtcacctcctagctgactcaaat
oTV34	gcggaaacgtcgttcacacggggtcattatagcgatttttttcg
oTV35	ctcactatagggtcgacaagtgatgtgaacgacgtttccg
oTV36	gatacatccgctttggaacgtatttttcccacacgcatcc
oTV37	cgtgtgggaaaaatacgttccaaagcggatgtatcggtgaaa
oTV38	cagctaggaggtgactgagccccaccgaacaaacttacaaa
oTV39	agtttgttcggtggggctcagtcacctcctagctgactcaaat
oTV40	acgtcgttcacatcacttgtcgaccctatagtgagtcgtattaaaaagg

3.2. CLONING

In this thesis used cloning technics were golden-gate cloning and Gibson assembly cloning.

3.2.1. Protocol for Golden-gate cloning

This protocol for golden gate cloning is based on protocol published in Bio-protocol (Vad-Nielsen et al., 2016.) with slight changes.

Plasmids used for cloning are pJOE9734.1 and pJOE9732.4 (Toymentseva and Altenbuchner, 2018). Plasmids content has been previously shown. The goal of cloning was to insert sgRNA into plasmid. Methods used to confirm successful transformations were blue-white screening and colony PCR.

The first step is to decide where in the genome of *B. thuringiensis* we want to introduce double-strand break. When place in genome is selected, pick 20 base pairs to be sgRNA. To choose the best possible sgRNA *GPP sgRNA designer* software developed by Broad institute has been used. To finish sgRNA, input suitable overhang on 5' end of oligos. The procedure is shown in Figure 8.

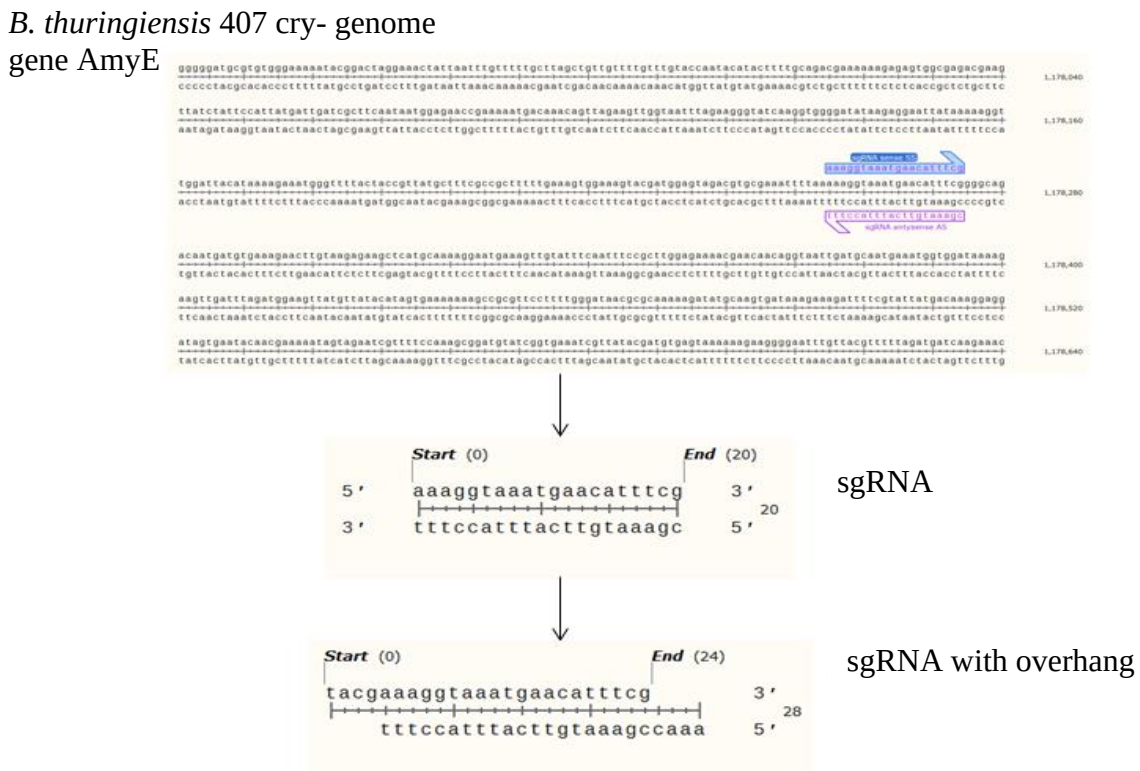


Figure 8. Designing of sgRNA with overhang.

Before insertion of sgRNA into the plasmid, the necessary step is annealing of sgRNA oligos (sense and antisense oligonucleotides). The reaction system is shown in Table 6. The procedure contains two steps. First, denaturation of the mixture at 95 ℃ for 5min using T100^{TM} Thermal Cycler (Bio-Rad Ltd., Richmond, USA). Subsequently, place mixture on the lab bench and let them slowly anneal until tubes reach room temperature.

Table 6. Reaction system for annealing sgRNA oligos.

Component	Volume
Sense sgRNA oligo	1 µL
Antisense sgRNA oligo	1 µL
10X T4 ligase buffer	2 µL
Milli-Q water	16 µL

Assembling of sgRNA and plasmids was performed in individual PCR tube in the thermal cycler. Restriction enzyme and ligase used for this cloning were purchased from Thermo Fisher Scientific and it was T4 DNA ligase and Eco31I restriction enzyme. Assembly mixture content is shown in Table 7. and incubation conditions are shown in Table 8.

Table 7. Reaction system during annealing of sgRNA into pJOE9734.1 or pJOE9732.4.

Component	Volume
Plasmid DNA	0.16 µL (100 ng)
Annealed sgRNA	1.00 µL
Eco31I	1.00 µL
T4 DNA ligase	1.00 µL
T4 DNA ligase buffer	2.00 µL
Milli-Q water	14.84 µL

Table 8. Incubation conditions during annealing of sgRNA into pJOE9734.1 or pJOE9732.4

Conditions	
10 cycles	5 min at 37°C 10 min at 22°C
Hold for	30 min at 37°C
Hold for	15 min at 75°C
Keep at	4°C

Product has been transformed (chemical transformation) to *E. coli* JM109 and plated on LB agar with antibiotic kanamycin (35 μg mL^{-1}), IPTG and X-Gal (IPTG and X-Gal have been placed on top of LB agar and left to dry). IPTG and X-Gal have been used to induce blue-white screening of bacterial colonies (white colonies are successful transformants, while blue one is not). After one day of incubation at 37 ℃ white colonies have been tested with colony PCR. Positive colonies have been picked and overnight culture have been made. The plasmid has been purified using plasmid DNA purification kit (NucleoSpin Plasmid, Macherey-Nagel) and put in freezer at -20 ℃. Scheme of protocol is shown in Figure 9.

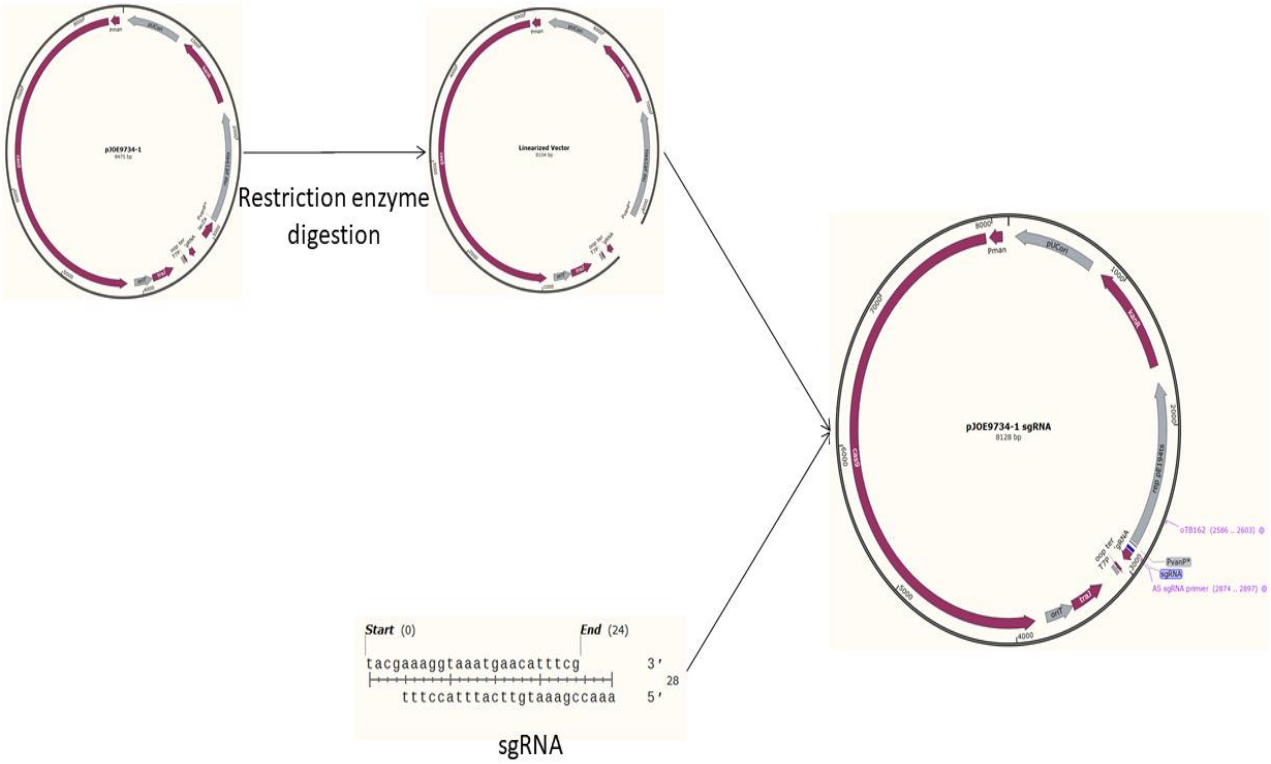


Figure 9. Scheme of Golden-gate cloning protocol.

3.2.2. Protocol for Gibson assembly

In this thesis, three different systems for Gibson assembly protocol have been used. To simulate process and design primers SnapGene (GSL Biotech LLC) software have been used. The first application was to develop plasmid from pJOE9734.1 that contain promotor (hyper-spank promotor), gene coding for GFP protein, and transcription terminator. A not necessary part of plasmid was Cas9 protein, so it was excluded. Also, to try to confirm activity of mannose promoter two different plasmids were required to be built (with and without Pman promoter). Scheme of protocol is shown in Figure 10.

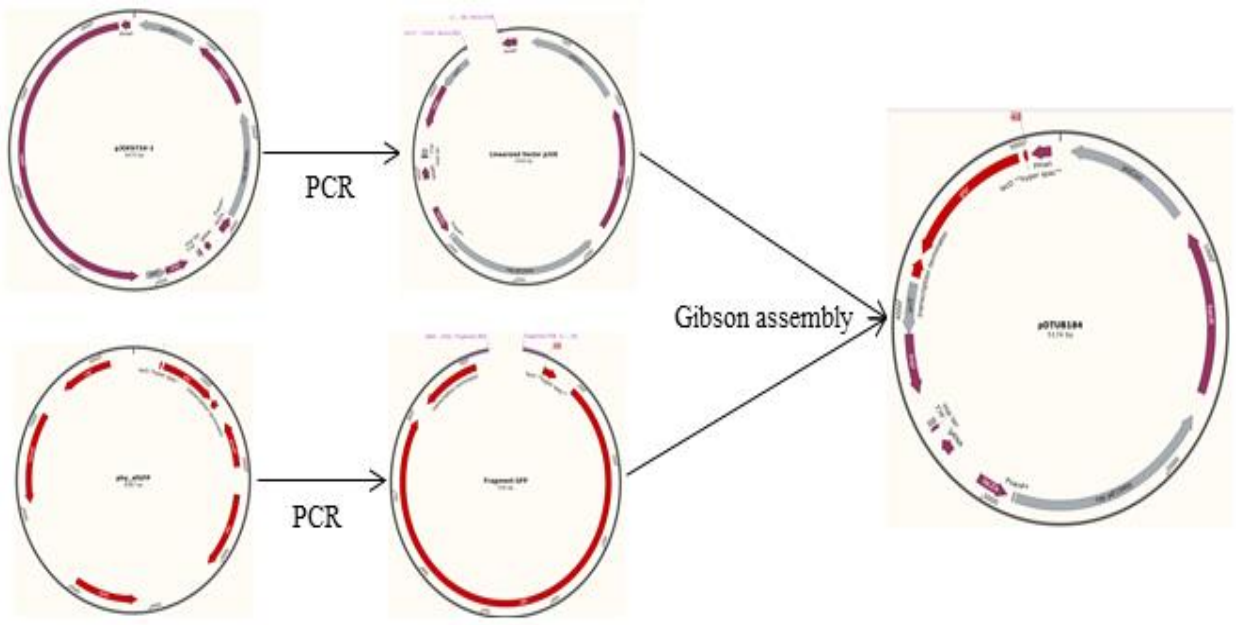


Figure 10. Scheme of Gibson assembly protocol

Plasmid backbone has been multiplied from pJOE9734.1 using PCR reaction and primers oTV01 and oTV02. To exclude Pman promoter then primers oTV01 and oTV05 have been used. GFP fragment has been multiplied from phy_GFP using PCR reaction, and oTV03 and oTV04. In another case without Pman promoter oligos oTV03 and oTV06 have been used. Polymerase used in this thesis was Q5 High-Fidelity DNA Polymerase (New England BioLabs). Protocol for PCR reaction can be found in Table 9.

Table 9. Example of PCR reaction system and thermocycling conditions. *****-annealing temperature that depends on primers sequence, **-extension temperature that depends on fragment size

Reaction system		**Thermocycling Conditions**	
Components	**Volume**	**Step**	**Time and Temp**
5X Q5 Reaction Buffer	4µL	Initial denaturation	30 sec at 98℃
10mM dNTPs	2µL	35 cycles	5-10sec at 98℃
10μM Forward Primer	1µL		10-30sec at 50-72℃*
10μM Reverse Primer	1µL		20-30sec/kb**
5-15 Template DNA	1µL	Final extension	5 min at 70℃
Q5 High-Fidelity Polymerase	0.2µL	Keep at	4℃
Milli-Q water	10.8µL		

When clear bands were confirmed with agarose gel-electrophoresis, PCR product has been digested with DpnI restriction enzyme. Afterwards, the PCR product was purified with PCR product purification kit. To calculate exact volume of reaction system the mass of fragments has been measured using the NanoDrop instrument. Example of Gibson assembly reaction system can be found in Table 10. Assembly protocol is one step incubation in thermocycler at 50 ℃ for 60 min. The following incubation was chemical transformation into *E. coli* JM109 strain. Grown colonies have been checked with colony PCR, stereo microscope (to check fluorescence), and plasmid digestion with restriction enzymes.

Table 10. Gibson assembly reaction system. X – represent the volume of total amount of fragments.

	Recommended Amount of Fragments Used for Assembly	
	2-3 Fragment Assembly	4-6 Fragment Assembly
Total amount of fragments	0.02-0.5 pmol→ (X µL)	0.2-1.0 pmol→ (XµL)
Gibson assembly master mix	10 µL	10 (µL)
Milli-Q water	20-X (µL)	20-X (µL)
Total Volume	20 µL	20 µL

Second, try to develop plasmids using pJOE9734.1 and pJOE9732.4 (containing sgRNA previously made with golden-gate cloning) as a backbone, and insert in three

fragments. Two of them are homologues regions of genome of *B. thuringiensis*, and the third fragment has promoter, gene coding for GFP protein, and transcription terminator. Primers oTV17-oTV28 have been used.

Third, try to develop plasmids using pJOE9734.1 and pJOE9732.4 (containing sgRNA previously made with golden-gate cloning) as the backbone, and insert in two fragments. Fragments are homologues regions of *B. thuringiensis* genome. Premiers oTV29-oTV40 have been used for PCR reaction.

In second and third Gibson assembly system the same protocol as for first has been used. Furthermore, in effort to have higher efficiency of assembly, overlap PCR has been performed to connect fragments and then have just two fragments assembly. In Table 11. is shown example of overlap PCR reaction system and thermocycling conditions.

Table 11. Example of overlap PCR reaction system and thermocycling condition that is done in two separate steps. *-there can be two fragments and more. X- sum of everything added in the system.

First Reaction system		**First Thermocycling Conditions**	
Components	**Volume**	**Step**	**Time and Temp**
5X Q5 Reaction Buffer	8.0 µL	Initial denaturation	30 sec at 98℃
10mM dNTPs	4.0 µL		5-10sec at 98℃
Fragment with overlap*	3.0 µL	14 cycles	10-30sec at 50-72℃
Q5 High-Fidelity Polymerase	0.4 µL		20-30sec/kb
Milli-Q water	40-X µL	Final extension	30 min at 37℃
		Keep at	4 ℃
Second Reaction system		**Second Thermocycling Conditions**	
Components	**Volume**	**Step**	**Time and Temp**
5X Q5 Reaction Buffer	2.0 µL	Initial denaturation	30 sec at 98℃
10mM dNTPs	1.0 µL		5-10sec at 98℃
10μM Forward Primer	2.5 µL	35 cycles	10-30sec at 50-72℃
10μM Reverse Primer	2.5 µL		20-30sec/kb
Q5 High-Fidelity Polymerase	0.1 µL	Final extension	5 min at 70℃
Milli-Q water	2.0 µL	Keep at	4 ℃
First reaction system	40 µL		

3.3. TRANSFORMATION

3.3.1. Chemical transformation of *E. coli*

Protocol for the chemical transformation of *E. coli* contains two separate steps. First is production of chemical competent cells, and then is chemical transformation.

First, a single colony has been picked to make overnight culture in LB (Lennox) medium. Inoculate 1 mL of overnight culture into 100 mL of fresh LB (100-fold). Grow till OD is between 0.3 - 0.4. Aliquot volume into two 50 mL plastic centrifuge tubes and cool on ice for 15 min. Centrifuge tubes at 2700 r.p.m. for 15 min on 4 ℃. Remove the supernatant and put tubes back on ice. Add 16 mL of cold RF1 buffer and resuspend cells. Return the tubes to ice for additional 15 min. Pellet the cells by centrifuging at 2700 r.p.m. for 15 min on 4 ℃. Remove the supernatant and resuspend in 4 mL of cold RF2 buffer. After 15min on ice aliquot 200 µL o resuspended cells into each 1.5 mL microcentrifuge tube. Freeze tubes into liquid Nitrogen and store at -80 ℃.

For transformation unfreeze cells slowly on ice. Mix 100 µL of competent cells with an appropriate amount of DNA and leave on ice for 30 min. Heat shock the mixture in heat block at 42 ℃ for 1 min and return on ice for 3 min. Add 300 µL of fresh LB and incubate in Eppendorf shaker at 37 ℃ for 1.5 h on 800 r.p.m. Plate 200 µL on LB agar plates containing appropriate antibiotic.

3.3.2. Electroporation protocol

To transform Gram-positive bacteria like *B. thuringiensis* highly efficient assay like electroporation is needed. The protocol used in this study is based on electroporation protocol for large plasmids and wild-type strains of *Bacillus thuringiensis* (Peng et al., 2009).

This protocol contains five steps: the growth of bacteria, cell wall-weakening treatment, preparation of electrocompetent cells, electroporation, and recovery period.

First of all, streak out and grow bacteria overnight on plates, pick one single colony and inoculate in liquid BHI media. Let bacteria grow overnight (no more than 16 h of incubation). Dilute overnight culture in an appropriate volume of BHI (hundredfold) and incubate them on 30 ℃, 220 r.p.m. until OD_{600} is around 0.2-0.3. In this study OD (optical density) has been measured by spectrophotometer.

Following, bacteria cell wall-weakening treatment is performed. In this study, wall-weakening treatment has been done by sterile glycine solution. When the bacterial culture reaches OD 0.2-0.3 glycine solution has been added until glycine concertation was 3 % (w/v), and then incubated on 30 ℃, 220 r.p.m. for 1 h.

From this point further everything needs to be cool down and kept on ice. Preparation of electrocompetent cells starts with cooling down cells on ice for 15 min in 50 mL plastic centrifuge tubes. Centrifuge the cells for 15 min at 2700 r.p.m. on 4 ℃. Discard supernatant and resuspend them in 15 mL ice-cold electroporation buffer. Return the tubes to ice for 10 min and subsequently centrifuge them for 15 min at 2700 g on 4 ℃. Repeat the washing step two times. After last centrifugation resuspends the cells in an appropriate volume of electroporation buffer and dispenses cells in aliquot of 200 µL to 1.5 mL microcentrifuge tubes. Freeze tubes in liquid Nitrogen and store at -80 ℃.

Electroporation was performed at 25 µF, 200 Ω and 3,0 kV in 0.2 cm electroporation cuvette using MicroPulser Electroporator (Bio-Rad Ltd., Richmond, USA). Before electroporation mix 100 µL of competent cells and 0.5 µg of plasmid DNA in Bio-Rad electroporation cuvette (Bio-Rad Ltd., Richmond, USA). Important notice, make sure that content is at bottom of the tube so pulse from electroporator can go safely threw content. Immediately after pulse, transfer content into liquid BHI and incubate for 2 h at 30 ℃ and 220 r.p.m. Spread 200 µL on the plates with antibiotic (chloramphenicol 10 µg mL^{-1}, and kanamycin 35 µg mL^{-1}) and incubate on 30 ℃ for 24-48 h.

To optimise this protocol deferent growing media have been used, but BHI showed best performances (data showed in results paragraph). Furthermore, different electroporation buffers, recovery periods, and concentrations of glycine have been used to optimise this protocol. Also, when transforming with plasmid pTB603 or pTB604 (chloramphenicol resistant) it was needed to add small concertation of antibiotic in recovery media (1 µg mL^{-1} of chloramphenicol). Scheme of protocol is shown in Figure 11.

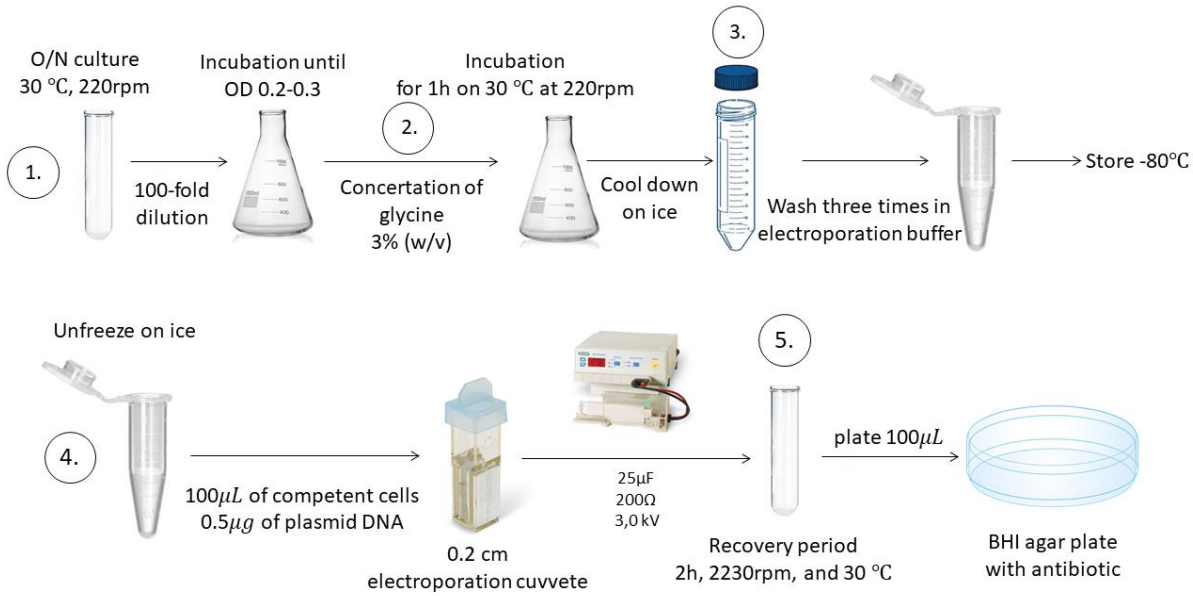


Figure 11. Scheme of electroporation protocol. The protocol contains five steps: 1. Growth of bacteria, 2. Cell wall-weakening treatment, 3. Preparation of electrocompetent cells, 4. Electroporation, 5. Recovery period.

3.3.3. Conjugation protocol

Another, older, but on the other hand more robust way of transforming bacteria is mating assay or conjugation. In this study chosen method of conjugation was solid conjugation previously described (Toymentseva and Altenbuchner, 2018).

In the mating assay, donor was gram-negative *E. coli* S17-1 (Simon et al, 1983). Recipients have been all gram-positive bacteria *B. thuringiensis, Paenibacillus sp., Bacillus sp.* closely relation to *B. simplex, Lysinibacillus fusiformis M5,* and *Bacillus velezensis FZB42.*

The donor is transformed (chemical transformation) with pJOE9734.1 and pDTUB184. Plasmids have origin of transfer (oriT), and a nickase that recognizes the oriT and interacts with conjugation transfer protein. The donor was grown overnight in LB with antibiotic kanamycin concertation 50 µg mL^{-1}. The donor has been washed three times to remove residual antibiotic, after last wash 2 mL of donor has been dispensed to 2.0 mL microcentrifuge tubes, centrifuged, and supernatant has been discarded. The donor has been resuspended with 0.2 mL of recipient, centrifuged again, and supernatant has been discarded. The mixture has been resuspended in 20 µL and then dropped on filter paper (0.22 µm nitrocellulose filters, MF-Millipore) positioned on LB agar plate supplemented with 0.2 % glucose and incubated overnight at 30 ℃. After 24 hours cell mass from filter paper was picked up and suspended in 1 mL of LB media. Appropriate dilution has been plated on LB agar plate containing 35µg mL^{-}

[1] of kanamycin (plasmid selection) and 12.5µg mL^{-1} of polymyxin (inhibits gram-negative bacteria). To calculate conjugation efficacy appropriate dilution of donor (LB agar) and recipient (LB agar with kanamycin) has been plated, respectively. Scheme of protocol is shown in Figure 12.

To make sure that grown colonies are indeed transformants and that they are producing GFP, few colonies have been picked up and grown overnight in media with antibiotic and plated again to compare morphology of ancestor and transformant.

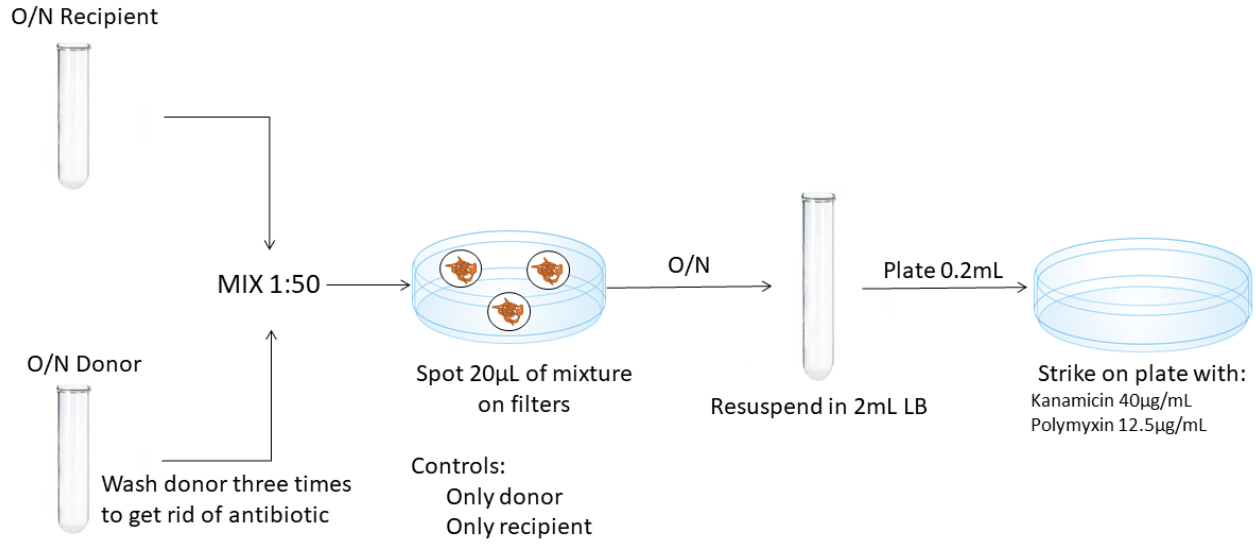


Figure 12. Scheme of conjugation protocol

3.3.4. Stereomicroscopy and Plate reader assay

Production of GFP and mKATE of every transformant has been tested using stereomicroscope and plate reader (Infinitive F200 PRO, TECAN).

Stereomicroscopy assay begins by making overnight cultures of transformants. Appropriate dilution of culture has been plated on LB agar plate with antibiotic and let grown 24-48 hours depending on strain. Colonies grown on plates have been observed under stereomicroscope (Carl Zeiss, Jena, Germany) equipped with a Zeiss CL 9000 Led light source, HE eGFP filter set (excitation at 470/40 nm and emission at 525/50 nm), HE mRFP filter set (excitation at 572/25 nm and emission at 629/62 nm) and an AxionCam MRm monochrome camera (Carl Zeiss, Jena, Germany).

As well, plate reader assay started by making the overnight culture of transformants containing appropriate concertation of antibiotic. Then 15 µL of each transformant was

transferred to a 24-well plate containing 1.5 mL of LB liquid media (100 dilutions). The plate has been placed in a plate reader where OD_{600} and fluorescence emission (excitation at 485/20 nm and emission 528/20 nm) have been measured every 15 min for 20 h. Plate reader has been set on 30 ℃ at 220 r.p.m. Data were collected and processed in Microsoft Excel.

3.4. ROOT COLONIZATION ASSAY

Protocol for root colonization assay was previously published in the Division of labour during biofilm matrix production by Dragoš (Dragoš, 2018).

Assay contains two separated process. First was growth of *Arabidopsis thaliana* plants, and then second bacterial root colonization.

Before seed incubation, the important step is seed surface sterilization using 2 % (w/v) sodium hypochlorite solution. After washing them for five times in sterile distilled water the seeds were placed on MS agar plates (Murashige and Skoog basal salt mixture, Sigma). After 3 days of incubation at 4 ℃, plates were placed at angle of 65° in a plant chamber on 21 ℃, 16 h light per day. After six days, homogenous seedlings were selected for root colonization. Seedlings were transferred into 24-well plates containing 270 µL of MSNg media per well (Dragoš, 2018). The content of MSN medium was prepared by weighting 0.026 g potassium dihydrogen phosphate, 0.061g dipotassium hydrogen phosphate, 2.09 g MOPS and 0.04 g magnesium chloride hexahydrate per 100 mL of demineralised water and adjusting the pH to 7.0. The base was autoclaved, cooled down to room temperature and supplemented with 0.1 mL of 0.7M calcium chloride, 0.05 mL of 100 mM manganese (ll) chloride, 0.1 mL of 1 mM zinc chloride, 0.1 mL of 2 mM thiamine, final concertation of ammonium chloride to 0.2 % and glycerol to 0.05 %.

Overnight bacterial culture was diluted to OD_{600} 0.2 and added into well with MSNg medium and plant. The sealed plates were incubated at rotary shaker at 28 ℃ for 24 h at 90 r.p.m. After the incubation, plants were washed 3 times with MSNg media and then transferred to a glass slide for imaging using Confocal Laser Scanning Microscopy. Scheme of the protocol is shown in Figure 13.

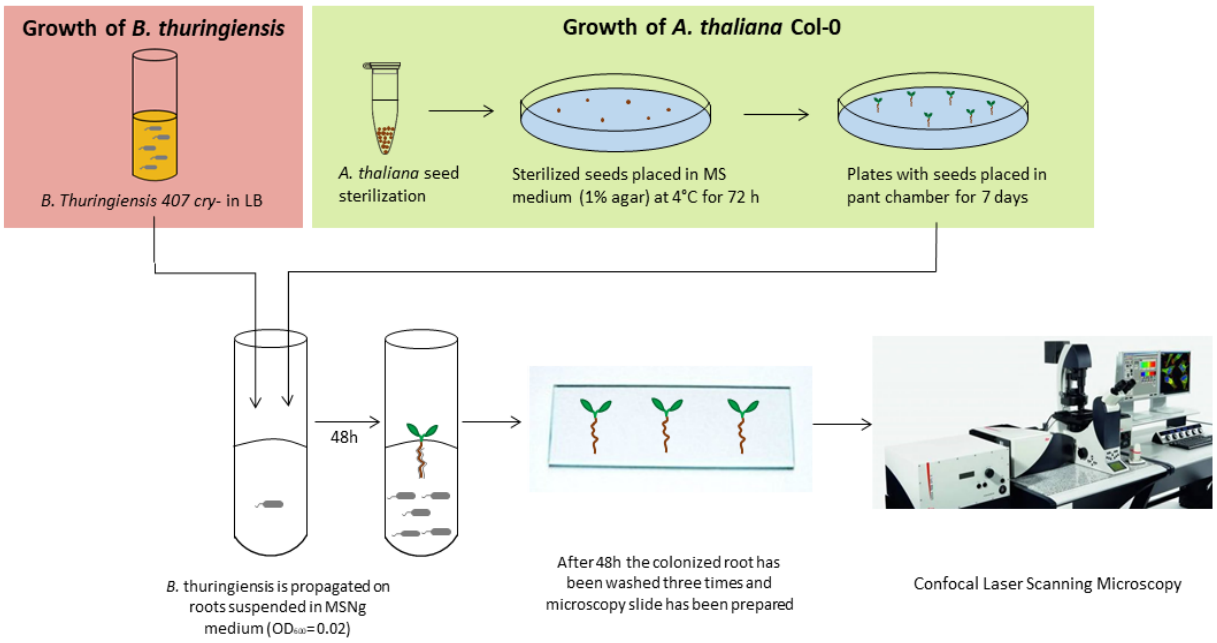


Figure 13. Scheme of root colonization assay

3.5. CONFOCAL LASER SCANNING MICROSCOPY

Confocal Laser Scanning Microscopy (CLSM) was used for visualisation of root colonization assay product. TCS SP8 Leica was employed in this thesis. CLSM is equipped with a Plan-Apochromat 63x/1.4 oil objective and as argon laser. Fluorescent reporter excitation was performed with an argon laser at 488 nm for green fluorescence and at 561 nm for red fluorescence. The emitted fluorescence was recorded 490-543 nm for GFP and 567-714 nm for mKate. Z-stack series with 1μm steps were acquired to generate multi-layer images.

3.6. PLASMID STABILITY ASSAY

Bacteria tend to lose plasmid DNA if there is no strict selection marker in media. Losing rate is individual for every strain and plasmid, and depend on multiple factors. In this thesis, plasmid stability was tested on *B. thuringiensis* and plasmids pTB603, pTB604, pDTUB184, and pDTUB185. Bacteria have been grown overnight in LB (Lennox) media with appropriate antibiotic (chloramphenicol 10 µg mL^{-1} or kanamycin 50 µg/mL). Culture has been diluted 100-fold in media without antibiotic (without selection marker) and incubates in shaker at 30℃ for 48 hours at 220 r.p.m. Samples have been taken from overnight culture, and after 10, 24, 34, and 48 hours of incubation. Appropriate dilution has been plated on plates with and without antibiotic. Colonies have been counted and data is shown in Results and discussion

4. RESULTS AND DISCUSSION

4.1. MOLECULAR CLONING

Cloning goals of the thesis are to make conjugative plasmid that has fluorescence reporters and to make vector with active CRISPR-Cas9 system for *B. thuringiensis* genome editing. Cloning methods used are very robust golden-gate cloning and Gibson assembly.

To construct conjugative plasmid DNA two different backbones have been used. Backbones are multiplied from pJOE9734.1 and they are very similar, the only difference is that one has mannose promoter (Pman), and the other doesn't have. Both conjugative plasmids that have GFP fluorescence reporter were successfully constructed. Positive results were obtained after transformed colonies have been tested with colony PCR and stereomicroscope. As well, purified and digested plasmid DNA gave on gel electrophoresis perfect bends. Plasmid digestion was performed using four restriction enzymes NheI, XbaI, ApaI, and BamHI. Pictures of PCR fragments used for Gibson assembly and colony PCR result you can find in Figure 14. Constructed plasmids are named pDTUB184 (that has mannose promoter), and pDTUB185 (doesn't have mannose promoter).

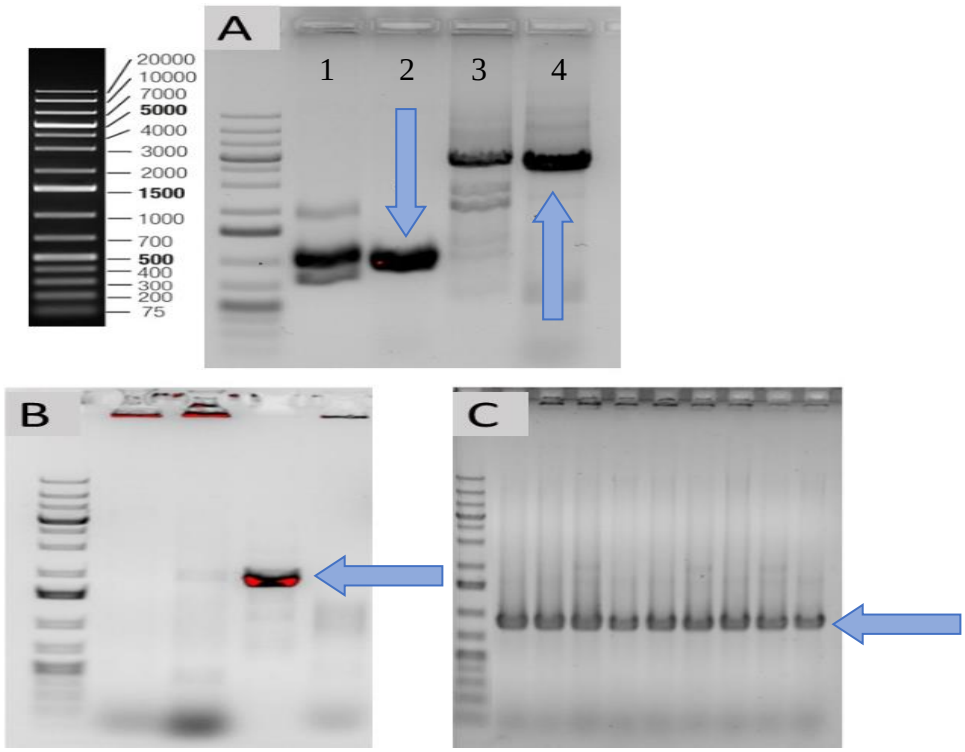


Figure 14. PCR amplification of (A) fragments used for Gibson assembly and colony PCR results for (B) pDTUB184 and (C) pDTUB185. With arrows are marked bands from which positive results are acquired.

As you can see on the gel in Figure 14., bands after PCR reaction and DpnI digestion are clean and yield is very high. In conclusion, perfect fragments, achieved in well number 2 and 4 are further used for Gibson assembly. Digestion with DpnI is performed for higher purity of PCR product (DpnI cut only methylated DNA, and with that lower the number of fouls positive results). New England BioLabs protocol for Gibson assembly said that if larger volume of unpurified PCR product is present, the Gibson assembly reaction could be inhibited. As well, for positive Gibson assembly reaction optimal quantities of DNA need to be added in system. Formula to calculate the concertation of pmols of each fragment for optimal assembly, based on fragment length and weight is showed in formulas (1), (2), and (3).

$$\text{pmol} = (\text{weight in ng}) \cdot 1000 / (\text{base pair} \cdot 650 \text{ daltons}) \quad (1)$$

$$50\text{ng of } 5000 \text{ pb dsDNA is about } 0.015 \text{ pmols} \quad (2)$$

$$50\text{ng of } 500 \text{ pb dsDNA is about } 0.15 \text{ pmols} \quad (3)$$

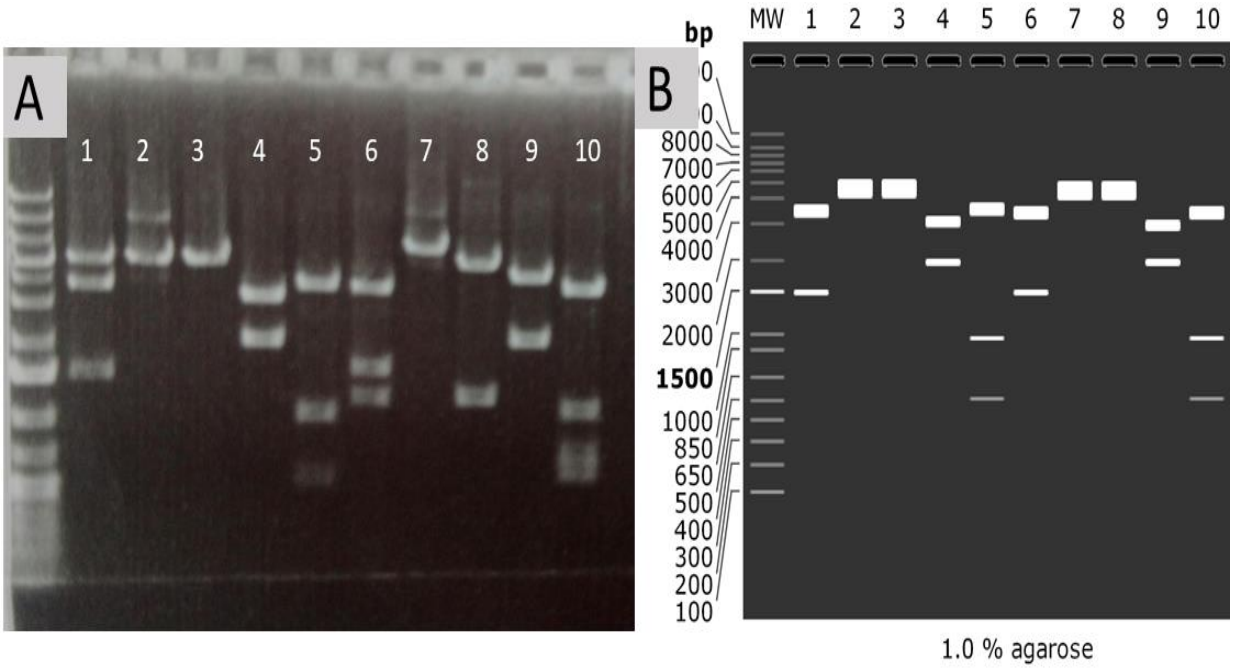
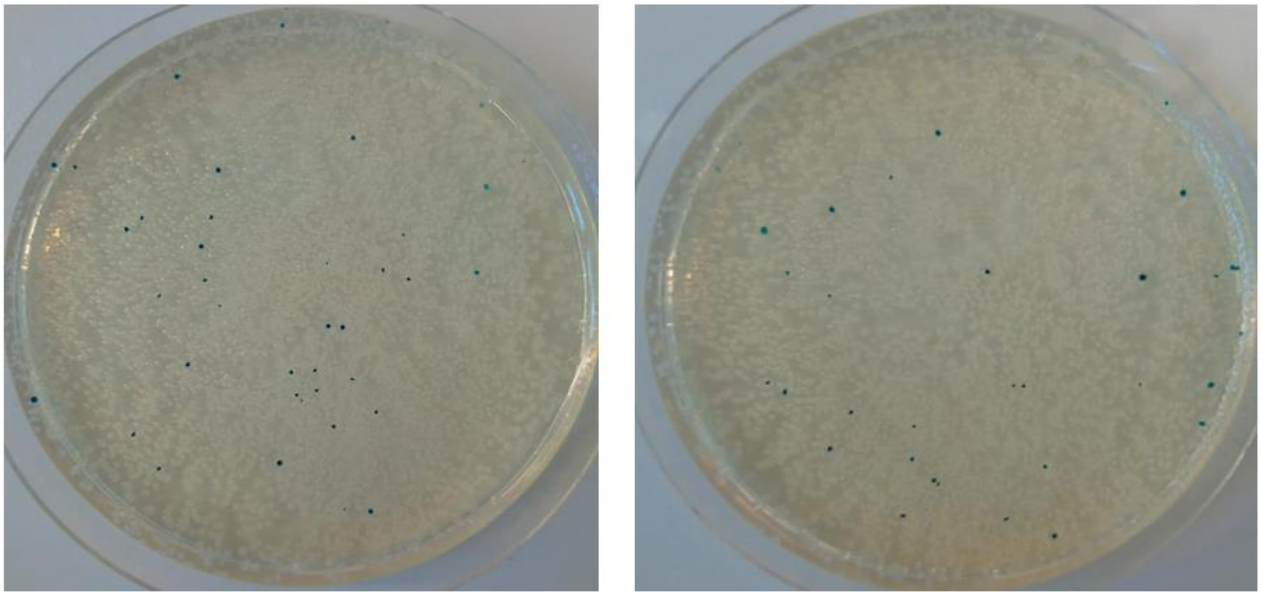


Figure 15. Restriction enzyme digestion of plasmids pDTUB184 (first five wells) and pDTUB185 (from well six till ten). A-plasmids digestion results, B- model made by SnapGene software.1., 6. well-digestion with XbaI and NheI; 2., 7. well-digestion with XbaI; 3., 8. well-digestion with NheI; 4., 9. well-digestion with XbaI and ApaI; 5., 10. well-digestion with NheI and BamHI.

Figure 15. shows plasmid digestion results and software made model. Furthermore, for plasmid pDTUB184 there is no difference between performed digestion and model. For plasmid pDTUB185 it seems that there is some mutation in plasmid DNA. When using NheI, instead of having just one spot where restriction enzyme cut plasmid, there is two of them. Consequentially, that is the reason why in well number 6., 8., and 10. higher number of bands is present. Nevertheless, the plasmid is same size, and mutation doesn't affect the expression of fluorescence. In conclusion, plasmid pDTUB185 can be still used. To check where is mutation, plasmid should be sent for sequencing. Plasmids pDTUB185 and pDTUB184 are further used for optimisation of transformation protocol.

In another cloning experiment, it was a task to make a plasmid that has active CRISPR-Cas9 system for *B. thuringiensis* genome editing. The plasmid used as the backbone is pJOE9734.1 (mannose promoter), and pJOE9732.4 (xylose promoter).

The first step was using golden-gate cloning to insert sgRNA into plasmid. As golden-gate cloning is very robust method this step was performed with high yield (Weber et al., 2011). As a method of visualisation of successful transformation blue-white screening has been performed (Brandt et al, 1991). Picture of plate with grown transformants has been shown in Figure 16. To confirm that white colonies are indeed transformants they were tested with colony PCR (Figure 17). Plasmids are then purified and further used for Gibson assembly.



pJOE9734.1 pJOE9732.4

Figure 16. Blue-white screening. Transformed *E. coli JM109* with plasmids pJOE9734.1 and pJOE9732.4 that contain sgRNA. Transformants have been plated on LB agar plate with antibiotic kanamycin, IPTG, and X-GAL.

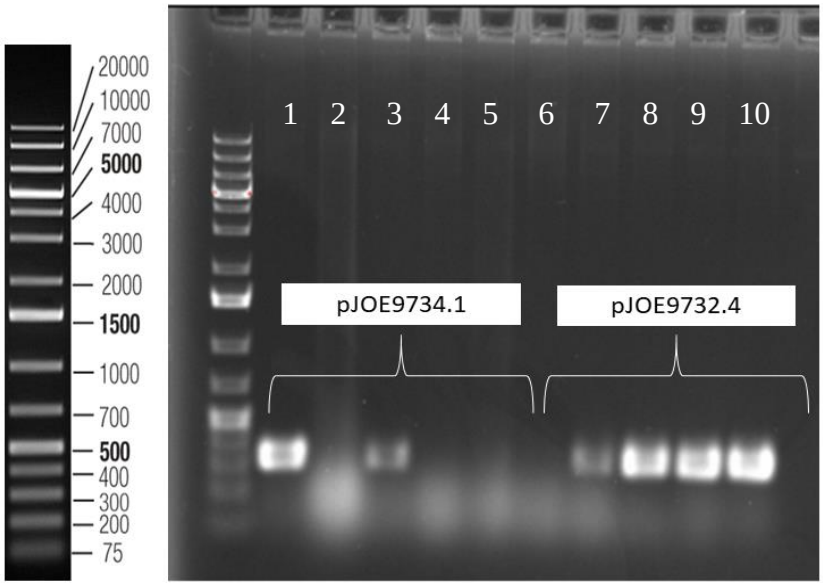


Figure 17. Colony PCR results for transformed *E. coli JM109* with plasmids pJOE9734.1 and pJOE9732.4 that contain sgRNA. In wells 1-5 are colony PCR results for plasmid pJOE9734.1, while for plasmid pJOE9732.4 colony PCR results are in wells 5-10. Best band are achieved in well 1 and 10 so these are used for further experiments.

Part of cloning that was unsuccessful was Gibson assembly of three and four fragments. Nevertheless, clean bands are achieved (Figure 18., figure A three fragments, figure D vector), and the same method as earlier described for Gibson assembly of two fragments was performed, nothing led to successful cloning. To troubleshoot problem different gel purification kit (Zymoclean^{TM} Gel DNA Recovery Kit) has been used, ultrapure agarose for gel purification has been used, different concentration (from pmols to nmols) and different ratios of fragments and backbone (3:1, 2:1, 5:1) has been used.

As well, overlap PCR of three (two homologues regions of *B. thuringiensis* genome, and gene for GFP), and two fragments (just homologues regions of *B. thuringiensis* genome) have been performed and even though there was troubleshooting threw PCR protocol (using different annealing time from 20-30s, and temperature from 60-72℃) clear bands on gel for gel purification has not been achieved.

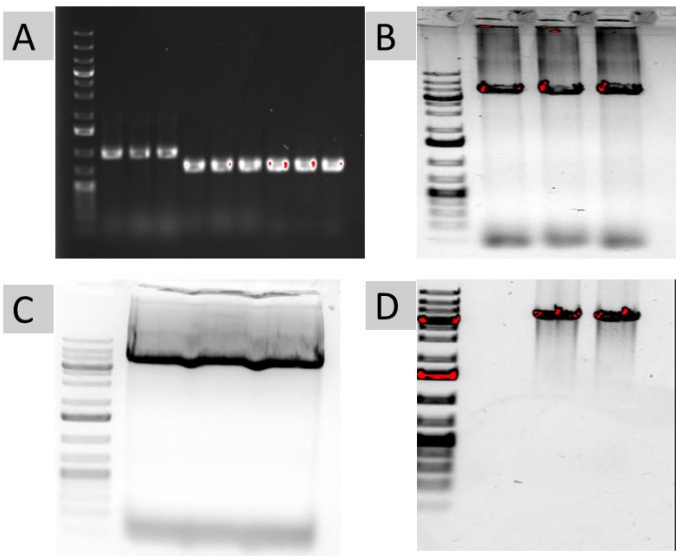


Figure 18. Electrophoresis of successful PCR reaction of (A) fragments (first three wells are GFP fragment, then next three left homologues regions of *B. thuringiensis* genome, and last three right homologues regions of *B. thuringiensis* genome) and (B) backbone (all three wells). (C) Backbone before DNA purification and (D) backbone after DNA purification.

To troubleshoot more in future, it would be better to use commercial competent cells, use a different strain of *E. coli*, change protocol from chemical transformation to electroporation (which should be more efficient) (Dower et al., 1998).

4.2. TRANSFORMATION

4.2.1. Electroporation

Electroporation has been proved to be very resourceful technic for the transformation of *B. thuringiensis* strains. At the beginning of protocol optimisation when using plasmids pTB603, and pTB604 there were no colonies. Literature showed that when having chloramphenicol resistance gene on plasmid it is necessary to have small concertation of chloramphenicol (1 µg mL^{-1}) in recovery medium to have successful transformants. Furthermore, growth conditions have been tested, and best performances have been achieved when growing conditions are at 30 ℃, and 220 r.p.m. for every step. In other papers (Sansinenea-Royano et al., 2010) different growing media have been used, therefore in this studies King's B media, LB media, Brain-heart-infusion (BHI) media, and Nutrient broth media have been tested. Nutrient broth showed lower performances than others and it was excluded. The best performances are achieved by BHI media, and it has been used furthermore.

As well as previously described (Peng et al., 2008) necessary step in electroporation protocol is wall-weakening treatment with glycine.

As it was emphasised before (Yi and Kuipers, 2016) every step after wall-weakening treatment should be cautiously performed on ice. For the preparation of electrocompetent cells, different buffers previously mentioned (Peng et al., 2008), have been tested. Electroporation buffer mentioned in Table 2. showed best performances for electroporation of *B. thuringiensis*. In electroporation process slow unfreezing of competent cells on ice had high influence on efficiency.

The best system for electroporation was to add 100 µL of competent cells and approximately 0.5 µg of plasmid DNA into electroporation cuvette. Tested conditions are 75, 100, 200 µL of competent cells, and 0.2, 0.5 and 1.0 µg of plasmid DNA. For electroporation conditions best performance has been achieved on 15.0 kV cm^{-1} voltage. Literature (Peng et al., 2008) said that even higher efficiency is on 20.0 kV cm^{-1}, but it was not possible to achieve due to the lack of equipment (0.1 cm electroporation cuvette).

One of the last things optimised was time of recovery (recovery period more than 2 h doesn't improve efficiency any more, tested 1, 1.5, 2, 2.5, 3h of recovery period), and antibiotic concertation on agar plates (for kanamycin 10, 25, 35, 50, 100, 200 µg mL^{-1}, and for chloramphenicol 5, 10, 20, 35, 50 µg mL^{-1}) that are inoculated with electroporation transformants (optimum was 35 µg mL^{-1} for kanamycin, and 10μg mL^{-1} for chloramphenicol).

Results of electroporation for *B. thuringiensis* 407 cry- ancestor and evolved strains transformed with pTB604 and pTB603 are shown in Figure 19.

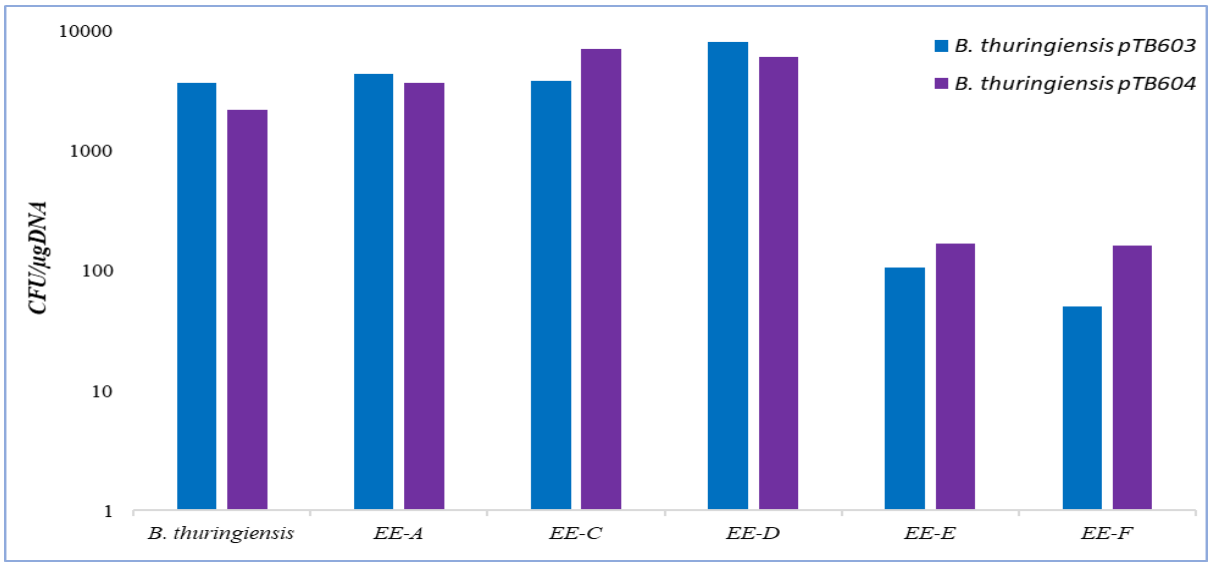


Figure 19. The efficiency of electroporation for *B. thuringiensis* ancestor and evolved strains transformed with pTB604 and pTB603.

It is clear from the results that are presented in Figure 19. that evolved strains A, C, and D show better transformation efficiency than the ancestor. On the other hand, evolved strains E, and F showed lower efficiency. Reasons for that cannot be said with any certainty, but with strains E and F there have been problems with manipulation of bacteria. They were forming aggregates or produce extracellular polysaccharides (EPS) (Krogstad, 2012), that probably disrupt the homogeneity of system, and correspondingly lower the number of produced competent cells.

In Figure 20. shown results present efficiency of *B. thuringiensis* 407 cry- transformation with different plasmids.

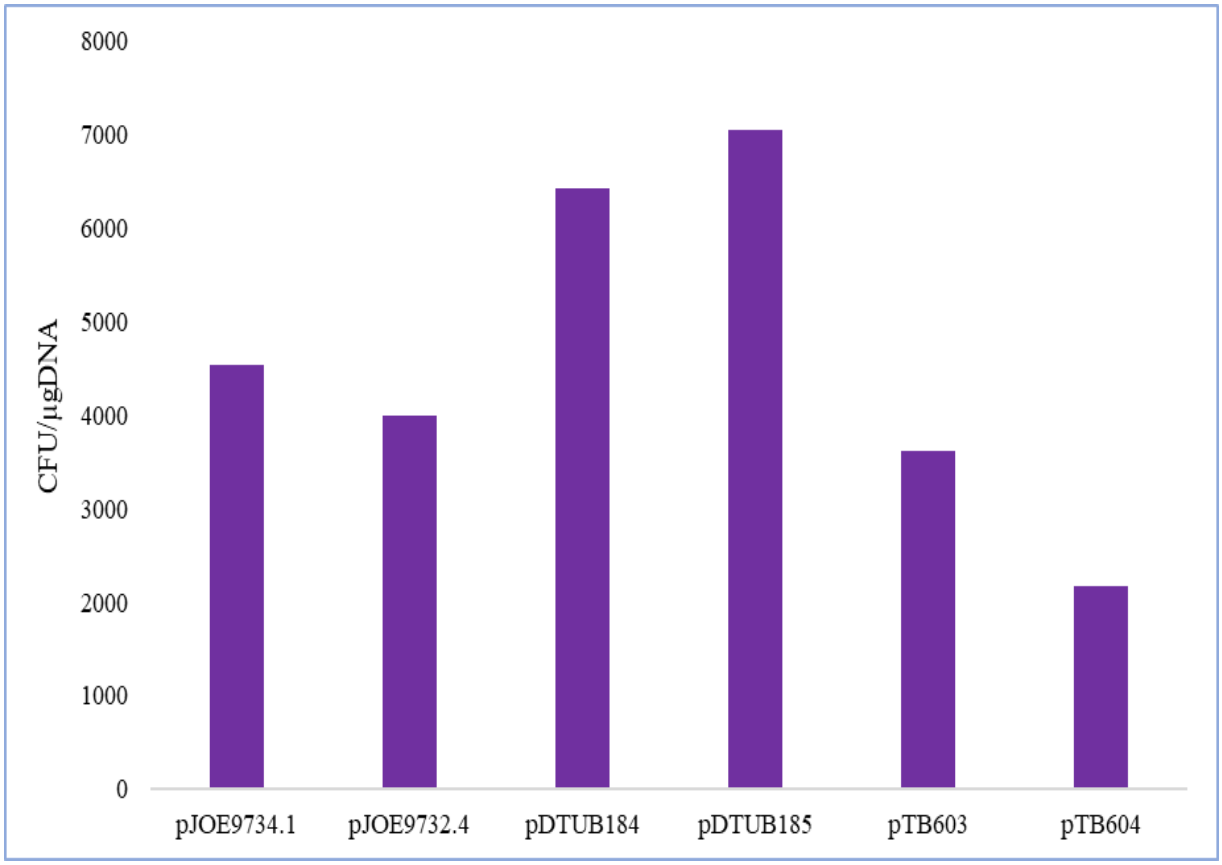


Figure 20. Efficiency of electroporation for *B. thuringiensis 407 cry-* ancestor strains transformed with pTB604, pTB603, pNW33n, pJOE9734.1, pJOE9732.4, pDTUB184, pDTUB185.

In this thesis, we attempted to transform by electroporation all other strains used in this thesis and label them with GFP fluorescence, but electroporation of these strains failed. Other strains are not well-explored strains, which usually means they are harder to manipulate. Additionally, these strains are undomesticated isolates that are not caring any mutation or have lost certain gene that makes them easier to cultivate and modify in the laboratory (Barchewitz

and Kovács, 2016). On that point, *Paenibacillus sp.* strain 040 hasn't been able to grow overnight to certain OD_{600} even though different media and temperature conditions have been tested.

However, it was surprising that other strains haven't been able to be transformed by electroporation, because it has been shown in this thesis, as well as in the literature, that other close related strains like *Bacillus cereus* could be transformed by electroporation (Turgeon et al., 2006). Nevertheless, this unsuccessful attempt to transform these strains by electroporation doesn't mean that with further optimisation of protocol it will not be achieved. Obviously, the optimisation of the electroporation protocol for these strains needs to be done more thoroughly. Bacteria needs to be looked at the genetic, cellular level, and right growth conditions need to be achieved to have so-called competence regime which result with successful transformation (Barchewitz and Kovács, 2016).

4.2.2. Conjugation

Another easy and robust method of plasmid transfer is solid conjugation. Conjugation or mating assay in this thesis has been performed for all Bacilli strains mentioned in Table 4. Almost all strains have been successfully transformed. Due to the cultivation problems, only strain that has not been successfully transformed was *Paenibacillus sp.* strain 040. This method was optimised. The first thing to check was ratio of cell number between donor and recipient that are put on mating. Tested ratios have been 70:1, 60:1, 50:1, 40:1. The best ratio has been 50:1 (50 cells of donor on one cell of recipient). Mating on plates with different media and with or without filters (0.22 µm nitrocellulose filters, MF-Millipore) have been tested. The best performance has been achieved with LB agar media supplemented with 0.2% (w/v) of glucose, and mating on filter paper.

In Table 13. you can find the number of transformants for every transformed strain. Strains have been transformed with pJOE9734.1 and pDTUB184. As you can see number of transformants for *B. thuringiensis* strain are pretty high. The number of transformants for *L. fusiformis* and *B. velezensis* are low, but at least it is constant and repeatable. Furthermore, improvement of the method should be accomplished with sonification as a method of disruption of aggregates formed while matting.

Table 13. Number of transformants (CFU mL^{-1}, CFU-colony forming unit) by conjugation of pJOE9734.1 and pDTUB184 from *E. coli* S 17-1 to *B. thuringiensis, Paenibacillus sp, B. simplex, L. fusiformis*, and *B. velezensis*

Strain	Plasmid	
	pJOE9734.1 (CFU mL^{-1})	pDTUB184 (CFU mL^{-1})
B. thuringiensis 407 cry-	4599	9090
Paenibacillus sp 037	490	110
B. simplex 050	130	969
L. fusiformis M5	13	14
B. velezensis FZB42	20	17

To calculate the efficiency of conjugation number of transformants has been divided with number of recipients. Results are shown in Table 14. As it was expected, the lowest efficiency is for *L. fusiformis* strain, even though *B. velezensis* is not far away. The biggest efficiency was for *B. thuringiensis* strain. Comparing to the literature (Toymensteva and Altenbuchner, 2018), this efficiency is very low, but there is a lot of space for progress and further optimisation of this method.

Table 14. The efficiency of conjugation for plasmids pJOE9734.1 and pDTUB184 from *E. coli* S 17-1 to *B. thuringiensis, Paenibacillus sp, B. simplex, L. fusiformis*, and *B. velezensis*

Strain	Plasmid	
	pJOE9734.1	pDTUB184
B. thuringiensis 407 cry-	$6.19 \cdot 10^{-6}$	$1.22 \cdot 10^{-5}$
Paenibacillus sp 037	$6.60 \cdot 10^{-7}$	$1.48 \cdot 10^{-6}$
B. simplex 050	$1.75 \cdot 10^{-7}$	$1.30 \cdot 10^{-6}$
L. fusiformis M5	$1.79 \cdot 10^{-8}$	$1.79 \cdot 10^{-8}$
B. velezensis FZB42	$2.69 \cdot 10^{-8}$	$2.24 \cdot 10^{-8}$

With this experiment, it was shown that conjugation, as a method of transformation, have grate potential if other transformation methods don't work. It is very robust and resourceful method that offers grate representability, stability, and repeatability. Comparing to other methods it is very cheap method that doesn't involve any machine or expensive chemicals. On the negative side it takes up to five days to get transformants and to confirm that obtained colonies are indeed successfully transformed. As well, method requires a lot of wet laboratory work.

4.2.3. Stereomicroscopy and Plate reader assay

To check if all transformants are indeed expressing fluorescence, transformants have been checked with stereomicroscopy and plate reader assay.

Stereomicroscopy has been performed on overnight grown colonies. In Figure 21. *B. thuringiensis* 407 cry- strain transformed with pTB603 and pTB604 is shown. In Figure 22. all other strains successfully transformed with pDTUB184 plasmid DNA are shown. Interestingly, bacteria's strain *L. fusiformis* after conjugation grow only on the edge of the agar plate near to plastic border.

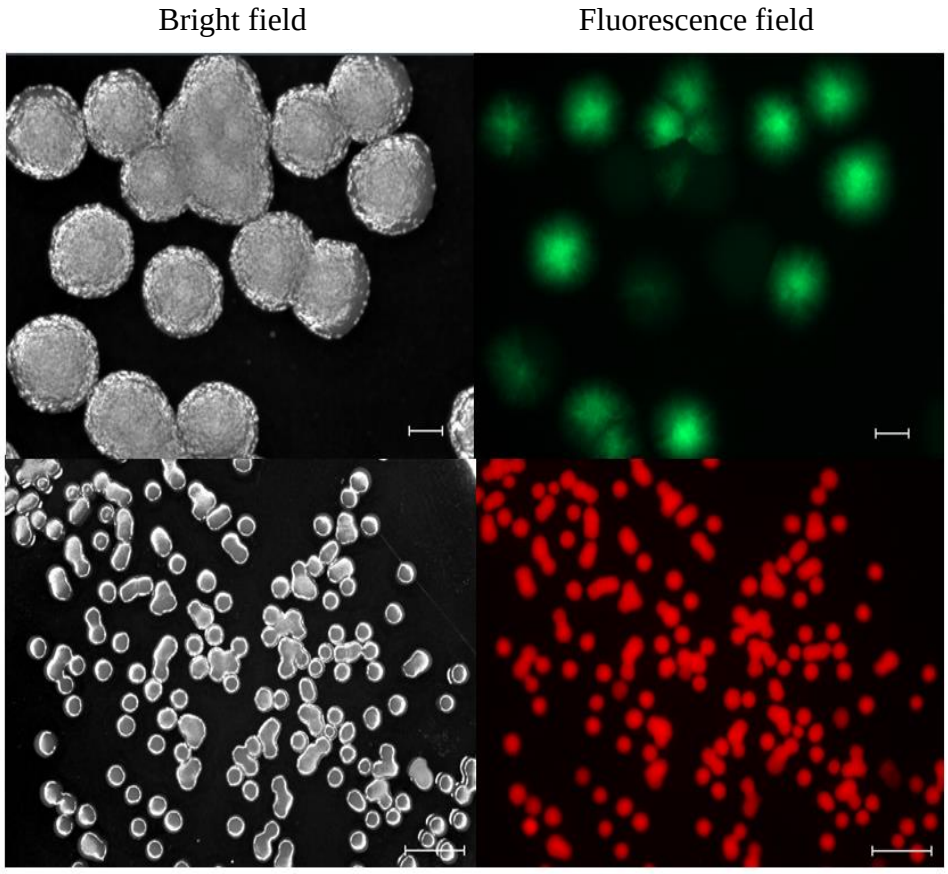


Figure 21. Stereomicroscope images of *B. thuringiensis* 407 cry- strain transformed with pTB603 and pTB604. Scale bar 5mm.

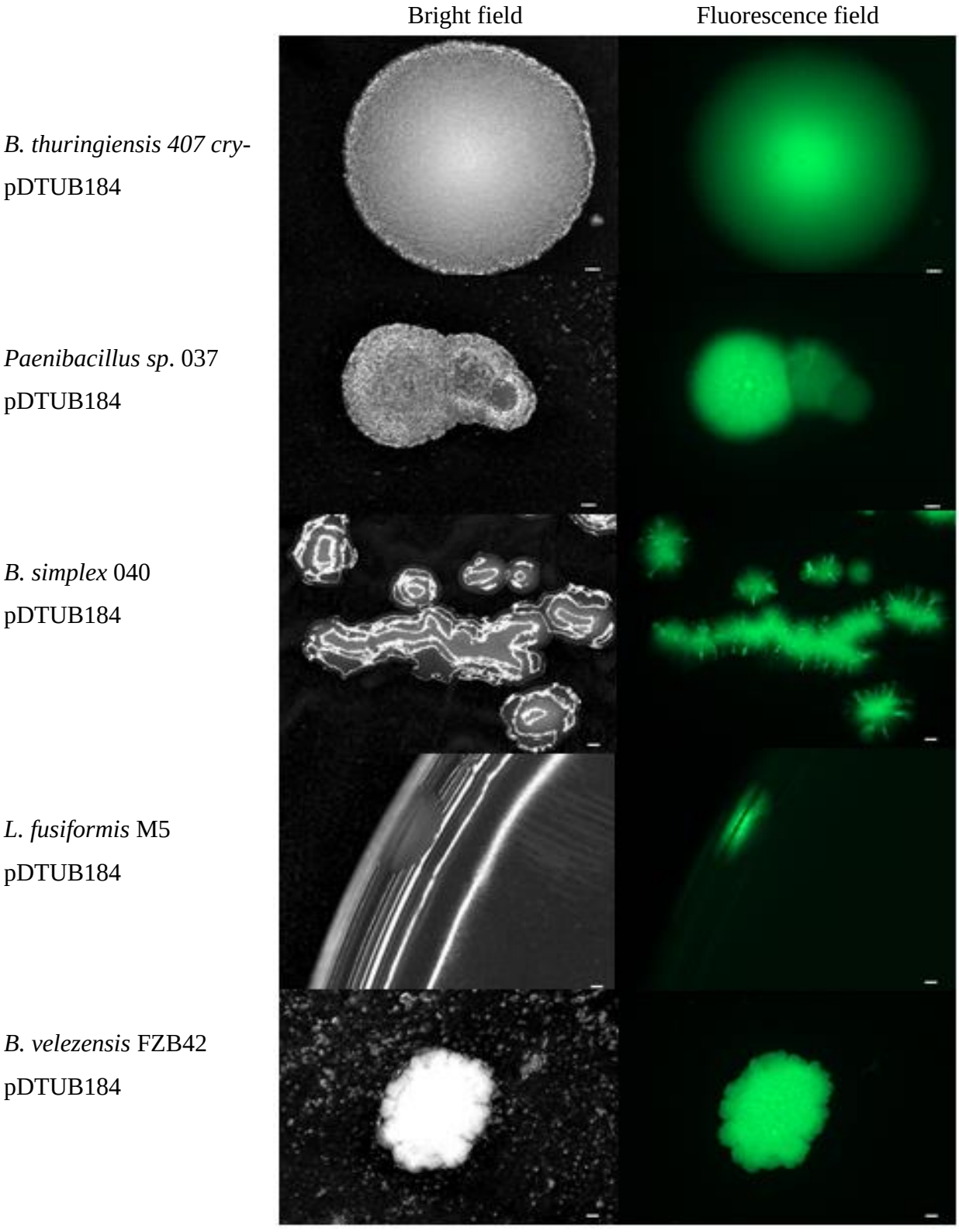


Figure 22. Stereomicroscope images of other strains successfully transformed with pDTUB184 plasmid DNA. Scale bar 5mm

Plate reader assay show us level of GFP expression in case of submersible conditions. As well, growth curve measured at OD_{600} has been made (Figure 23.). Sterile media has been used as control.

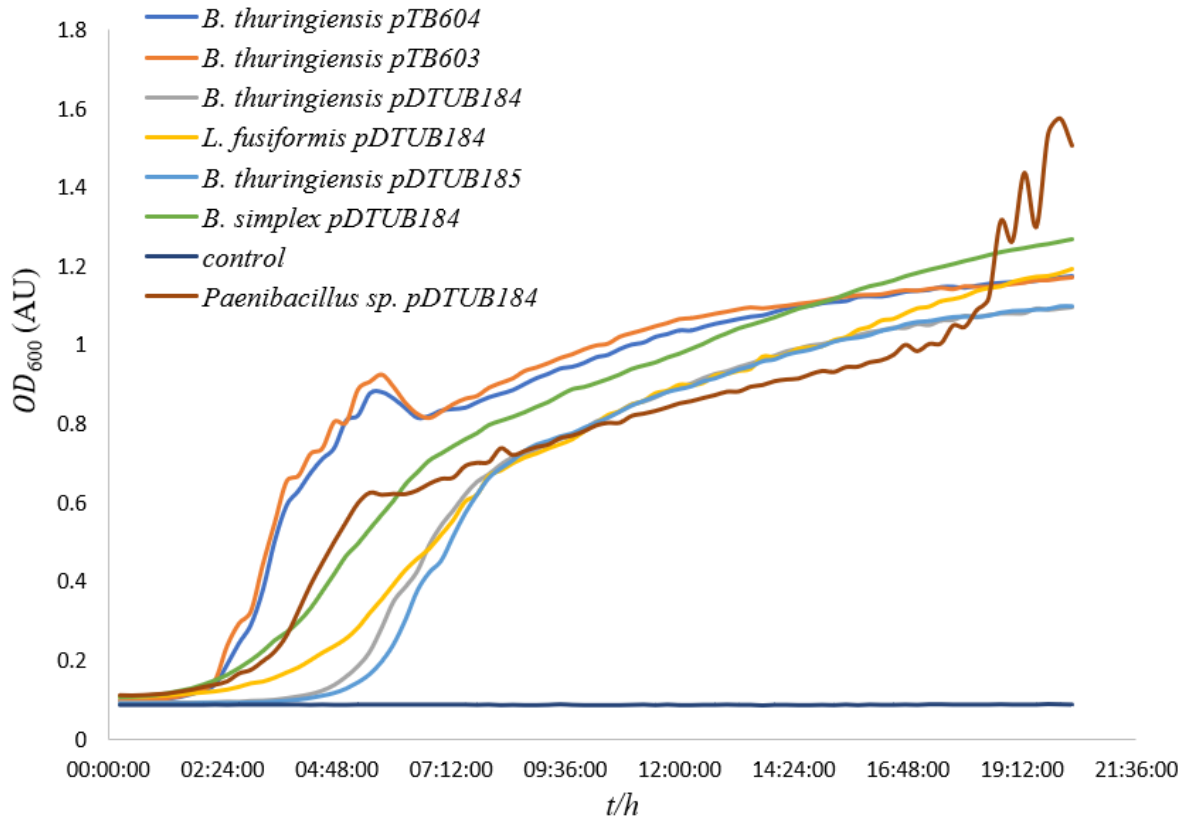


Figure 23. Growth curve made by plate reader. The control contains sterile media. Bacteria have been inoculated with 100-time dilution from overnight culture. Cultivation conditions has been 30℃, 220 r.p.m. in duration of 21 h in 24-well plate.

GFP fluorescence curve brings some expected and some not expected results (Figure 24.). In the graph, it is obvious that *B. thuringiensis* transformed with pTB603 show biggest production of GFP. Strains *L. fusiformis, B. simplex,* and *Paenibacillus sp.* are as well producing GFP but in lower amount. For some reason *B. thuringiensis* transformed with pDTUB184, and pDTUB185 doesn't produce any fluorescence in submersible growth. It can't be problem with strain because strain transformed with different plasmid is producing GFP. Additionally, it can't be problem with plasmid because other strains grown submersible contain pDTUB184, and they are showing fluorescence. Explanation should be found in other parts of plasmid like origin of replication ext., but they were out of scope of this thesis.

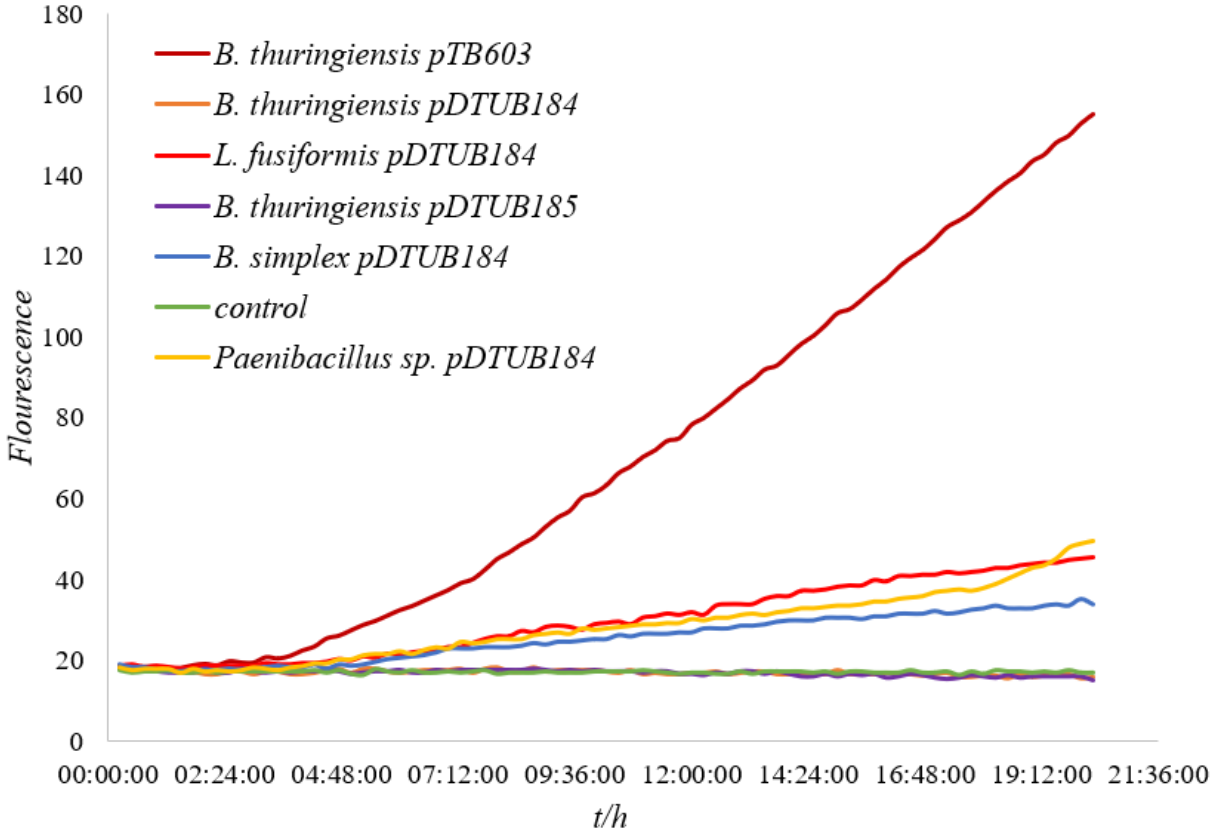


Figure 24. GFP fluorescence curve made using plate reader. The control contains just sterile media. Bacteria have been inoculated with 100-time dilution from overnight culture. Cultivation conditions has been 30℃, 220 r.p.m. in duration of 21 h in 24-well plate.

4.3. ROOT COLONIZATION ASSAY

B. thuringiensis is known as good root coloniser (Vidal-Quist et al., 2013). To analyse if transformed *B. thuringiensis* can colonise root in media with antibiotic, root colonization assay combined with confocal laser scanning microscopy has been performed. Images have been analysed with ImageJ by Fiji software.

The assay showed that *B. thuringiensis* is a really good root coloniser. The assay is repeatable and results are stable even dough literature says that there is possible death of plant due to the presence of antibiotic (Conte et al., 2009). Images in Figure 25. shown representable situation.

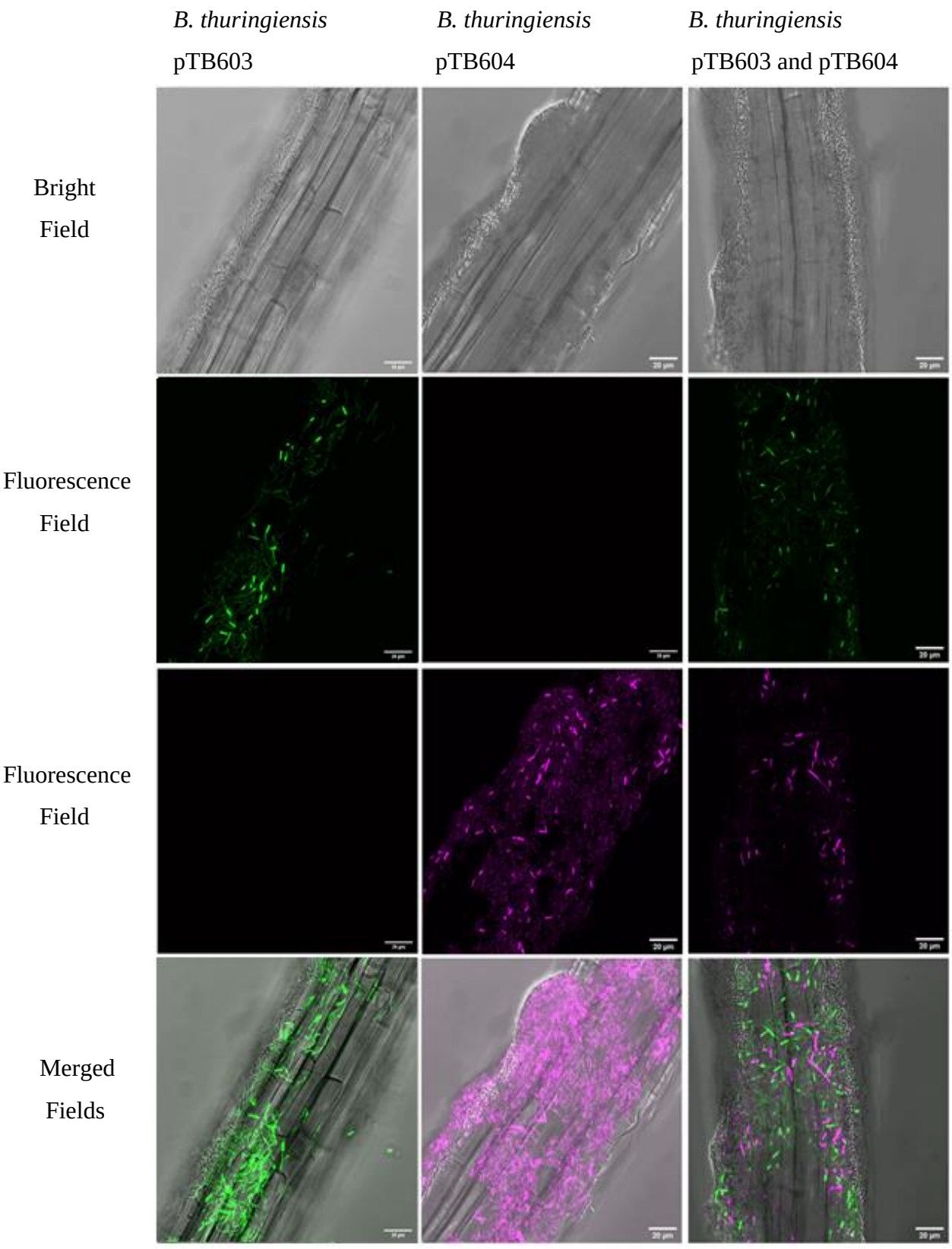


Figure 25. Root colonization assay of *B. thuringiensis* 407 cry- labelled with pTB603, and pTB604. The plant used for root colonization is *A. thaliana.* Pictures were taken with CLSM approximately near to the beginning of the root. Scale bar represents 20 µm.

4.4. PLASMID STABILITY ASSAY

It is well known that bacteria lose plasmid DNA if there is no selection marker (Friehs, 2004). Losing speed is individual for every strain (Friehs, 2004), and it needs to be confirmed with plasmid stability assay. Results are shown in Table 15. represent number of colonies grown on plates with and without antibiotic for *B. thuringiensis* strain transformed with pTB603, pTB604, pDTUB184, pDTUB185.

Table 15. Results of plasmid stability assay. Colonies have been grown overnight in media with antibiotic and transfered to media without antibiotic, and cultivated at 30 ℃, on 220 r.p.m. in a shaker. Samples have been taken after 10 h, 24 h, 34 h, and 48 h. Samples have been plated on LB agar plates with or without antibiotic. After 24 h of incubation grown colonies have been counted and they are represented as CFU mL^{-1} (CFU- colony-forming unit). CHL-chloramphenicol, KM-kanamycin

time	*B. thuringiensis pTB603* (CFU/mL)		*B. thuringiensis pTB604* (CFU/mL)	
	LB agar	LB agar + CHL	LB agar	LB agar + CHL
10h	$4.30 \cdot 10^7$	$4.50 \cdot 10^6$	$7.53 \cdot 10^7$	$3.05 \cdot 10^6$
24h	$4.71 \cdot 10^7$	$2.90 \cdot 10^6$	$3.07 \cdot 10^7$	$1.76 \cdot 10^6$
34h	$5.48 \cdot 10^7$	$1.38 \cdot 10^6$	$5.22 \cdot 10^7$	$4.80 \cdot 10^6$
48h	$3.72 \cdot 10^7$	$2.62 \cdot 10^6$	$3.53 \cdot 10^7$	$2.79 \cdot 10^6$
	B. thuringiensis pDTUB184		*B. thuringiensis pDTUB185*	
	LB agar	LB agar + KM	LB agar	LB agar + KM
10h	$3.96 \cdot 10^7$	$2.00 \cdot 10^5$	$9.49 \cdot 10^7$	$2.00 \cdot 10^5$
24h	$3.21 \cdot 10^7$	$8.65 \cdot 10^4$	$6.37 \cdot 10^7$	$2.95 \cdot 10^5$
34h	$7.50 \cdot 10^7$	$9.55 \cdot 10^4$	$3.79 \cdot 10^7$	$1.27 \cdot 10^5$
48h	$2.73 \cdot 10^7$	$5.28 \cdot 10^4$	$8.15 \cdot 10^7$	$1.16 \cdot 10^5$

In order to better understand results, the percentage of cells that still have plasmid DNA is shown in Figure 26.

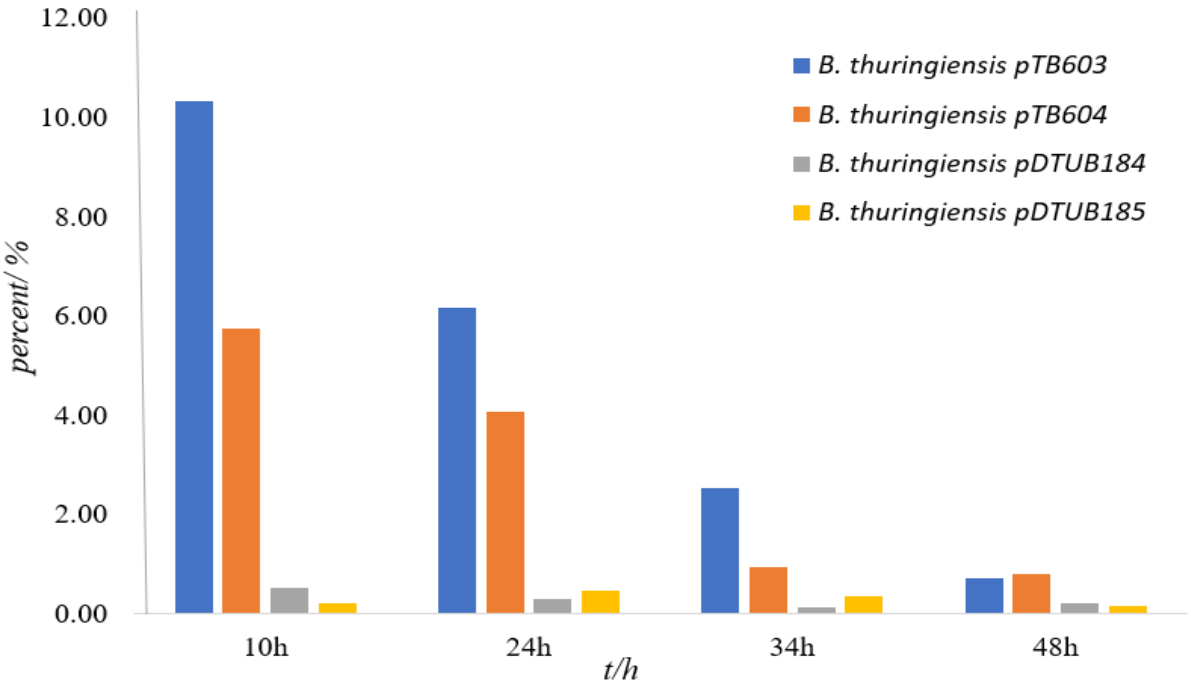


Figure 26. Percentage of cells that still have plasmid DNA in media without any selection marker. The percentage has been calculated by dividing the number of cells grown in media without antibiotic with number of cells grown in media with antibiotic and multiplied with 100.

From the graph, it is obvious that cells are losing plasmids in media without antibiotic. For cells with plasmids pDTUB184, and pDTUB185 lost in first 10h is significant, but afterwards, percent of cells that contain plasmid seems to be stable on 0.13-0,33 % of culture. For cells with plasmid pTB603 it seems that they are losing plasmid with constant rate and at the end it is around 0.7 % of culture that still has plasmid DNA. In comparison with cells transformed with pTB603, in first 10h cells transformed with pTB604 lose plasmid faster. Later, cells tend to lose plasmid slower, and at the end, after 48h of cultivation, culture with plasmid pTB604 have similar percentage of cells that still has plasmid DNA like culture transformed with pTB603.

Furthermore, it is interesting to see that plasmids pDTUB184 and pDTUB185 have similar losing rate, and quite different from plasmids pTB603 and pTB604. On the other hand, some similarities in losing rate can be seen for plasmids pTB603 and pTB604. These similarities are probably due to the fact that plasmids backbone for plasmid pTB604 and pTB603 are the same. Subsequently, plasmids pDTUB184 and pDTUB185 have the same backbone.

5. CONCLUSIONS

Based on the results obtained within this Thesis, the following can be concluded:

1. Two plasmids, pDTUB184 and pDTUB185, have been constructed successfully and both are suitable for the transformation of strains from order *Bacillales*.
2. Unfortunately, a plasmid with an active CRISPR-Cas9 system, that was intended to be used in *Bacillus thuringiensis 407 cry-* genome editing, has not been constructed due to many obstacles experienced during experimental part of this Thesis.
3. Electroporation protocol was successfully optimised and used as a part of the transformation procedure for *B. thuringiensis* strains.
4. Optimised conjugation protocol worked properly for all strains used in this Thesis (*Bacillus thuringiensis 407 cry-*, *Paenibacillus sp., Bacillus simplex, Lysinibacillus fusiformis M5,* and *Bacillus velezensis FZB42*).
5. All tested strains were transformed and easily detected due to co-expression of GFP gene and consequentially corresponding fluorescence. It is important to highlight that the transformed strains require antibiotic selection pressure (kanamycin or chloramphenicol) because otherwise they lose plasmid.
6. It is experimentally established that transformed *B. thuringiensis* 407 cry- pTB603 and *B. thuringiensis* 407 cry- pTB603 colonise roots of *Arabidopsis thaliana*, and is good candidate for plant-growth-promoting bacterium.

6. LITERATURE

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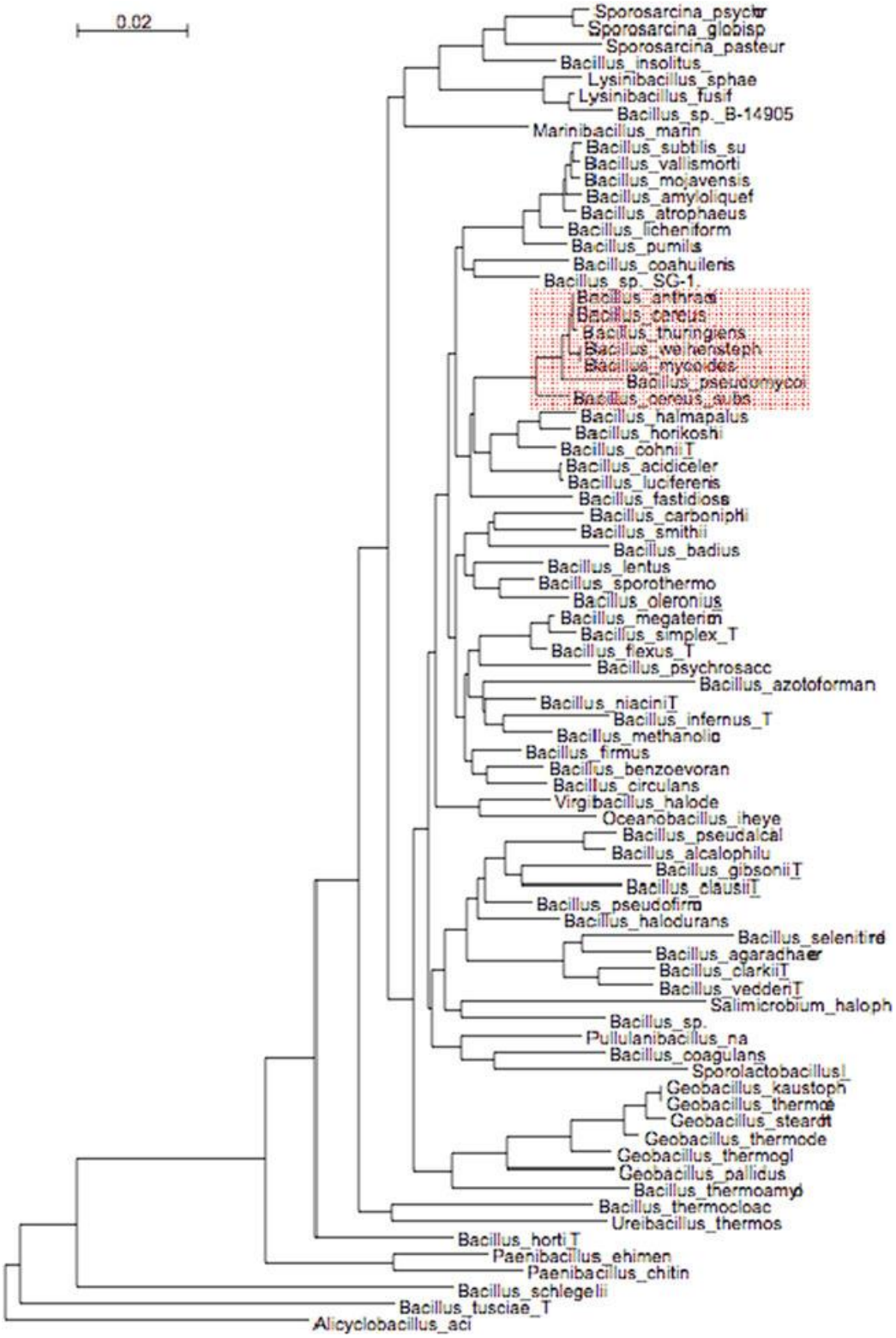
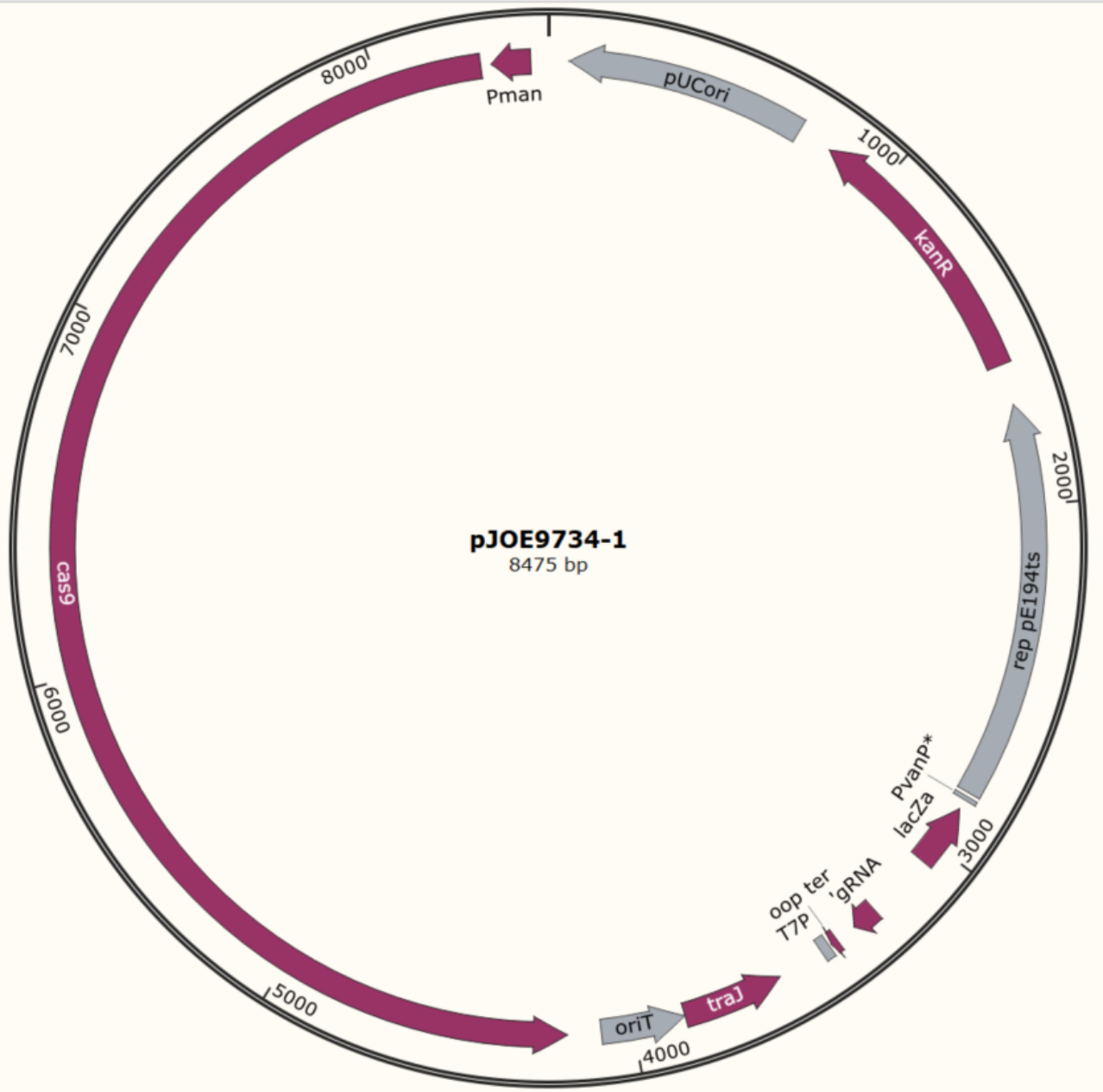
7. APPENDIX



Appendices 1. Phylogeny of species within the *Bacillus* genus, based on 16S rRNA sequence data. The *B. cereus* group members are boxed in red. The horizontal bar indicates a genetic distance of 0.02. Kindly provided by Økstad and Kolstø (Økstad and Kolstø, 2010).



Appendices 2. Plasmid pJOE9734.1 sequence and characteristic:

Cas9 - RNA-guided DNA endonuclease,

Pman - mannose-inducible promoter,

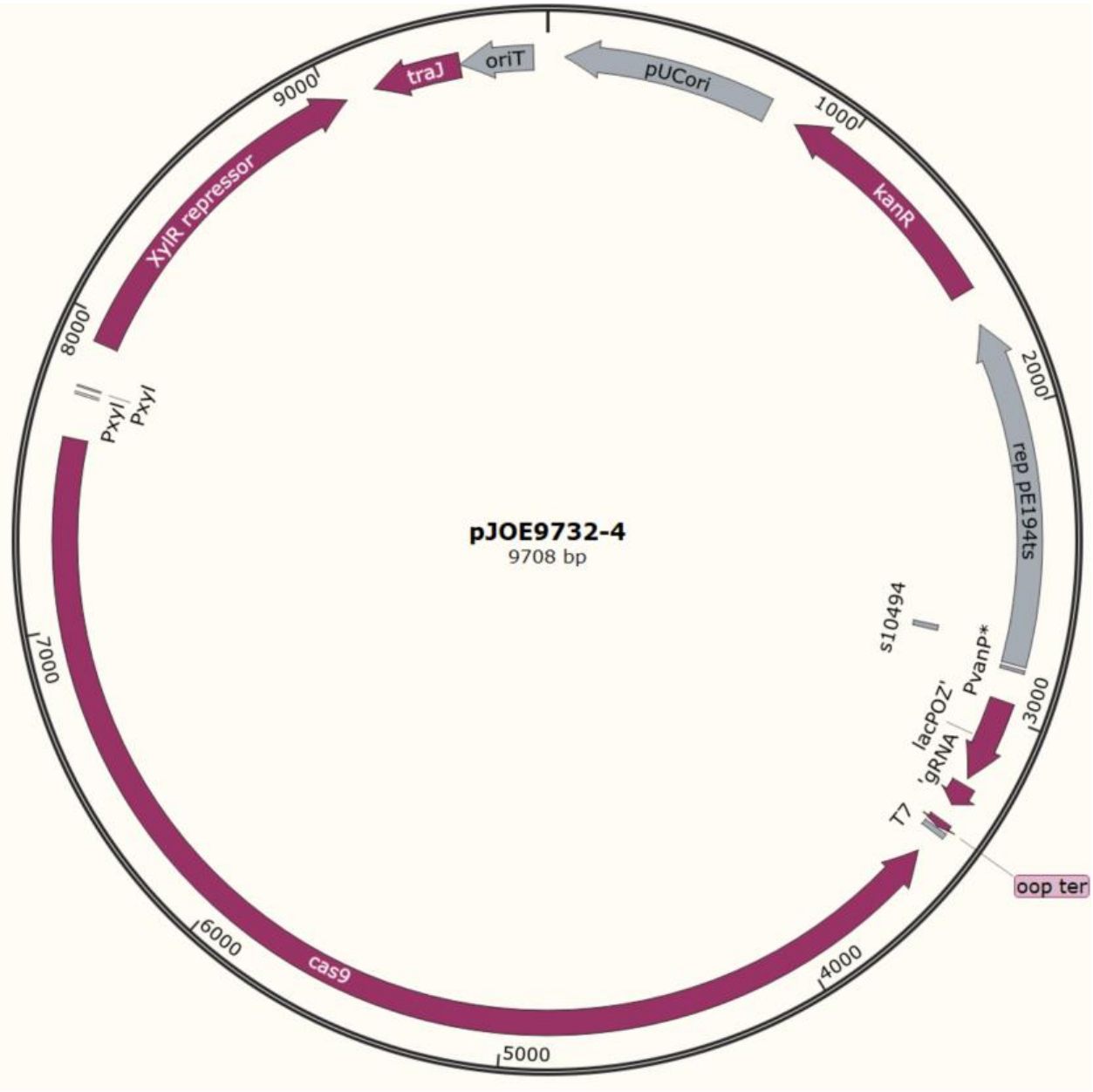
oriT - origin of transfer,

traJ - nickase recognizing oriT,

rep rep p194^{ts} - Origin of recombination in *Bacillus* cells,

pUCori - origin of replication in *E. coli*,

kanR - kanamycin resistance gene.



Appendices 3. Plasmid pJOE9732.4 sequence and characteristic:

Cas9 - RNA-guided DNA endonuclease,

P_{xyl}, XylR repressor- xylose promoter,

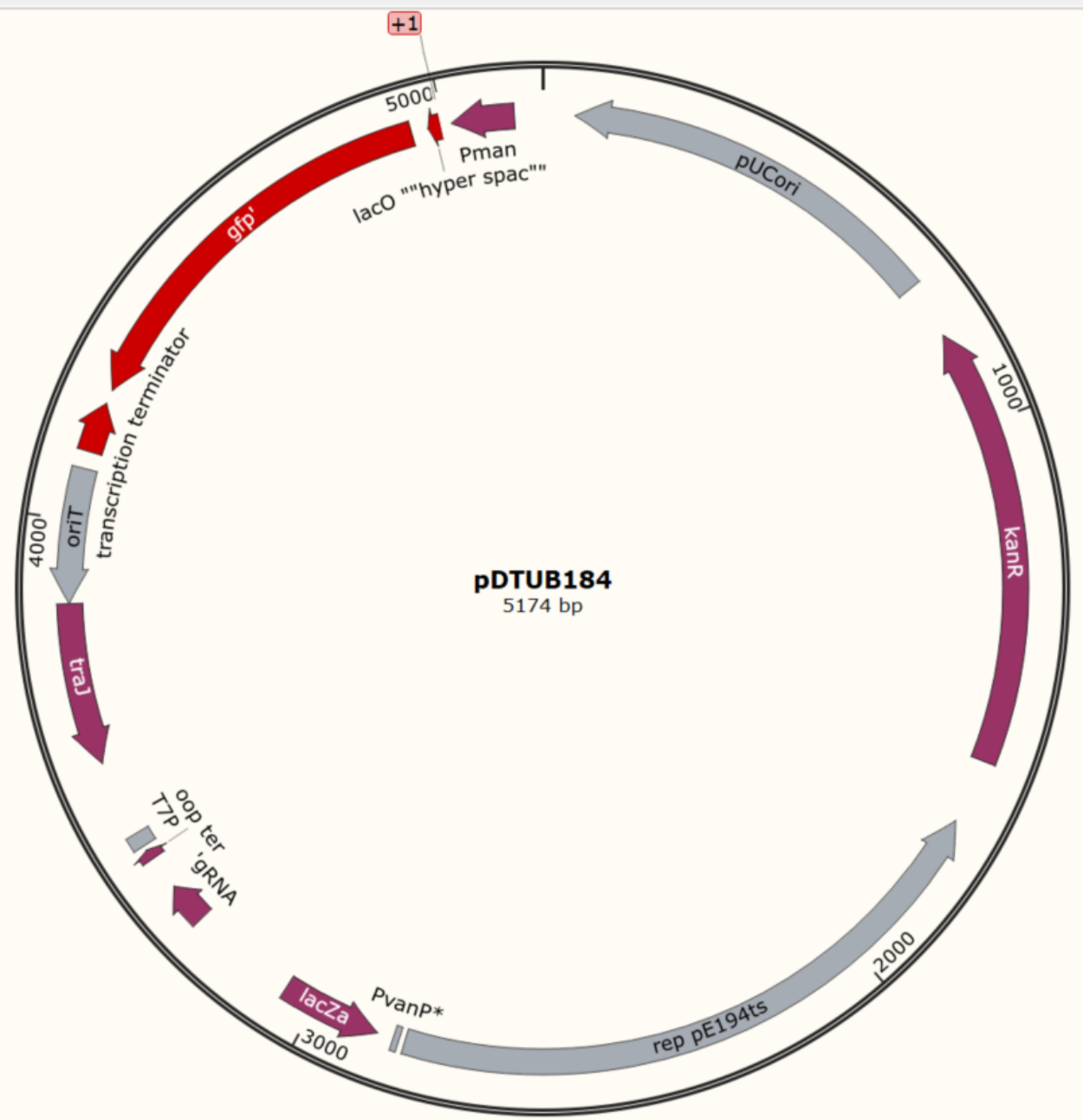
oriT - origin of transfer,

traJ - nickase recognizing oriT,

rep pE194^{ts}- origin of recombination in *Bacillus* cells,

pUCori - origin of replication in *E. coli*,

kanR - kanamycin resistance gene.



Appendices 4. Plasmid pDTUB184 sequence and characteristic:

gfp - GFP fluorescence protein,

lacO “hyper spac” - universal promoter,

Pman- mannose-inducible promoter,

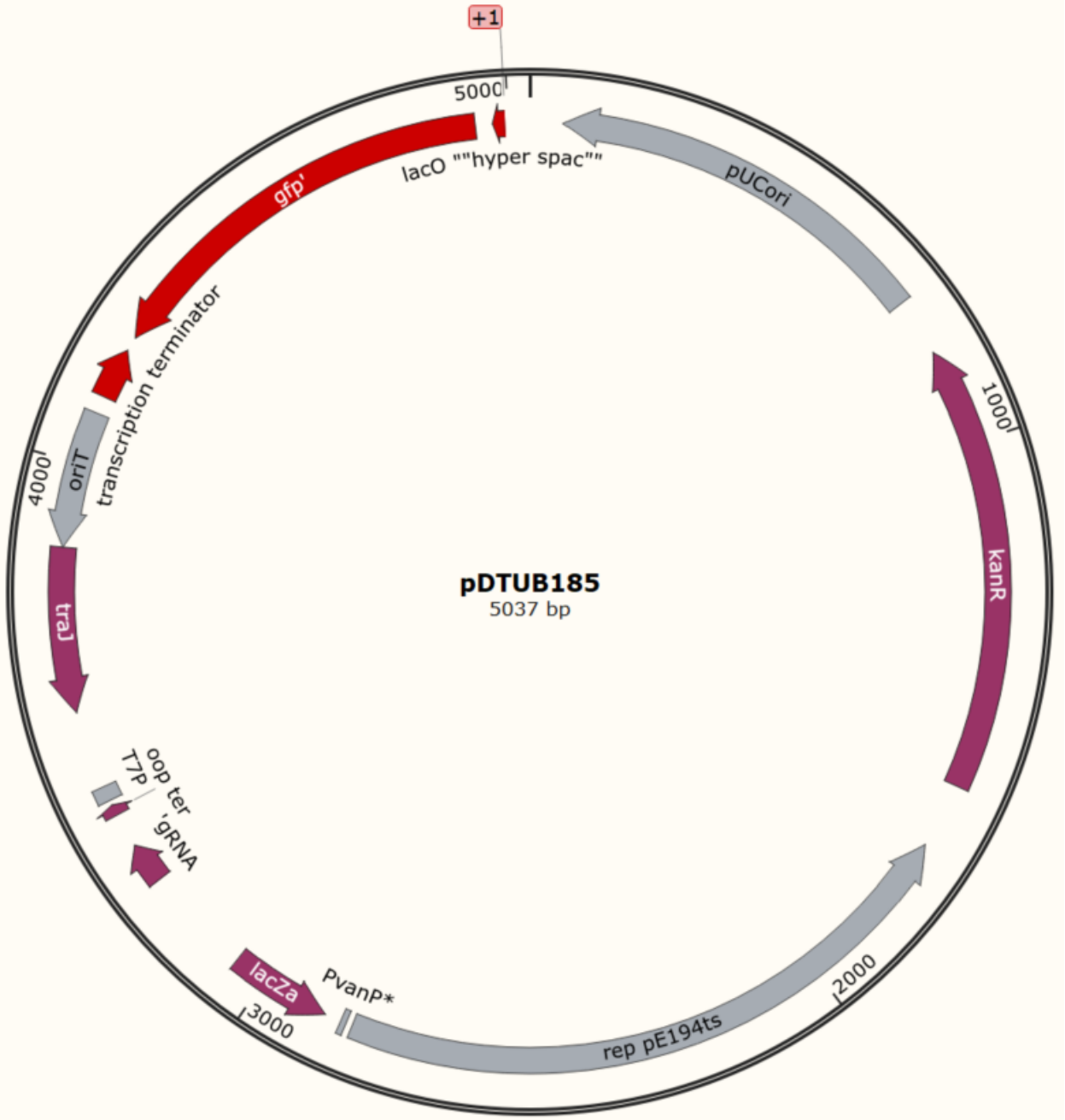
oriT - origin of transfer,

traJ - nickase recognizing oriT,

rep rep p194^{ts} - origin of recombination in *Bacillus* cells,

pUCori - origin of replication in *E. coli*,

kanR - kanamycin resistance gene.



Appendices 5. Plasmid pDTUB185 sequence and characteristic:

gfp - GFP fluorescence protein,

lacO “hyper spac”- universal promoter,

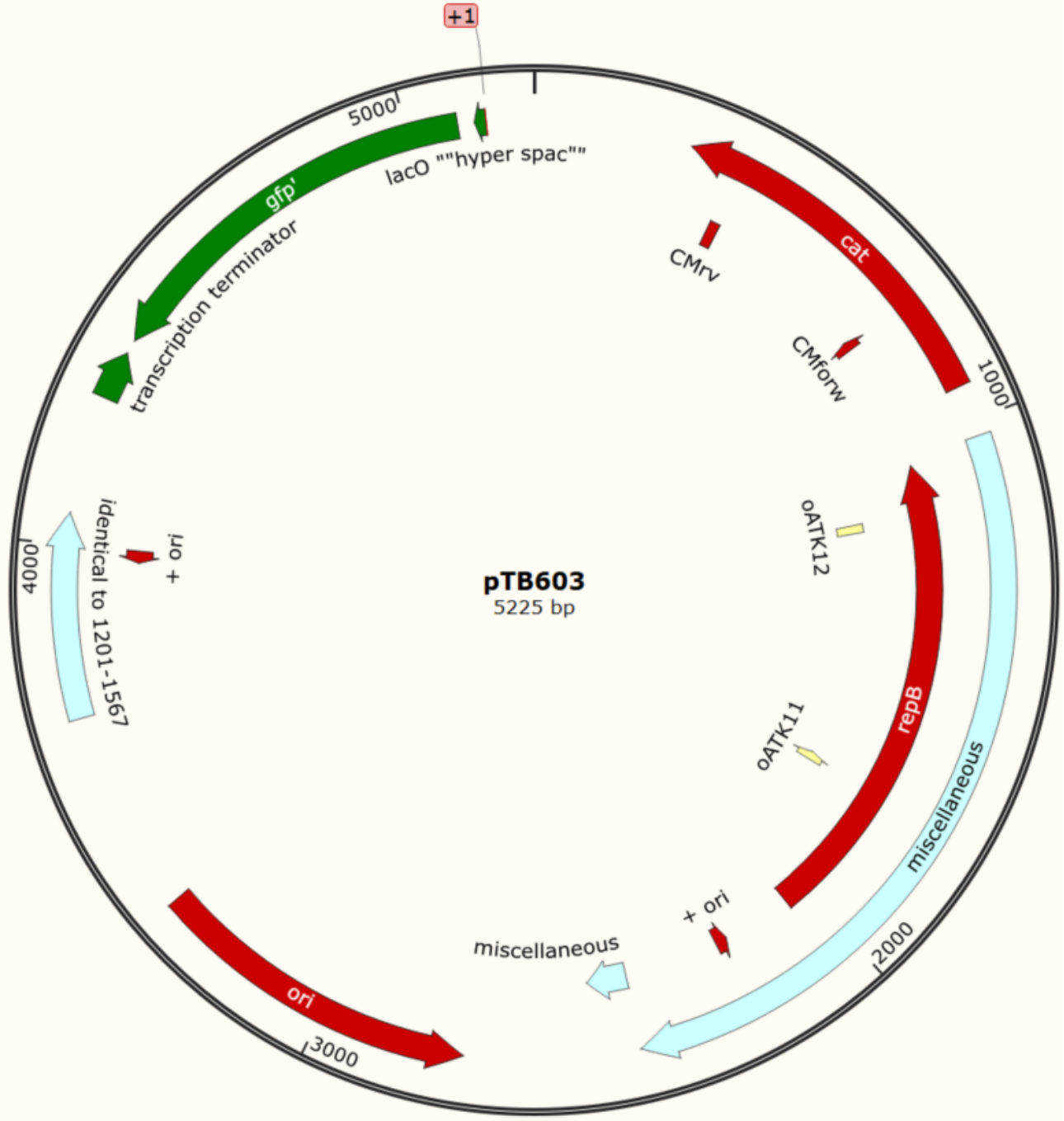
oriT - origin of transfer,

traJ - nickase recognizing oriT,

rep rep p194^{ts} - origin of recombination in *Bacillus* cells,

pUCori - origin of replication in *E. coli*,

kanR- kanamycin resistance gene.



Appendices 6. Plasmid pTB603 sequence and characteristic:

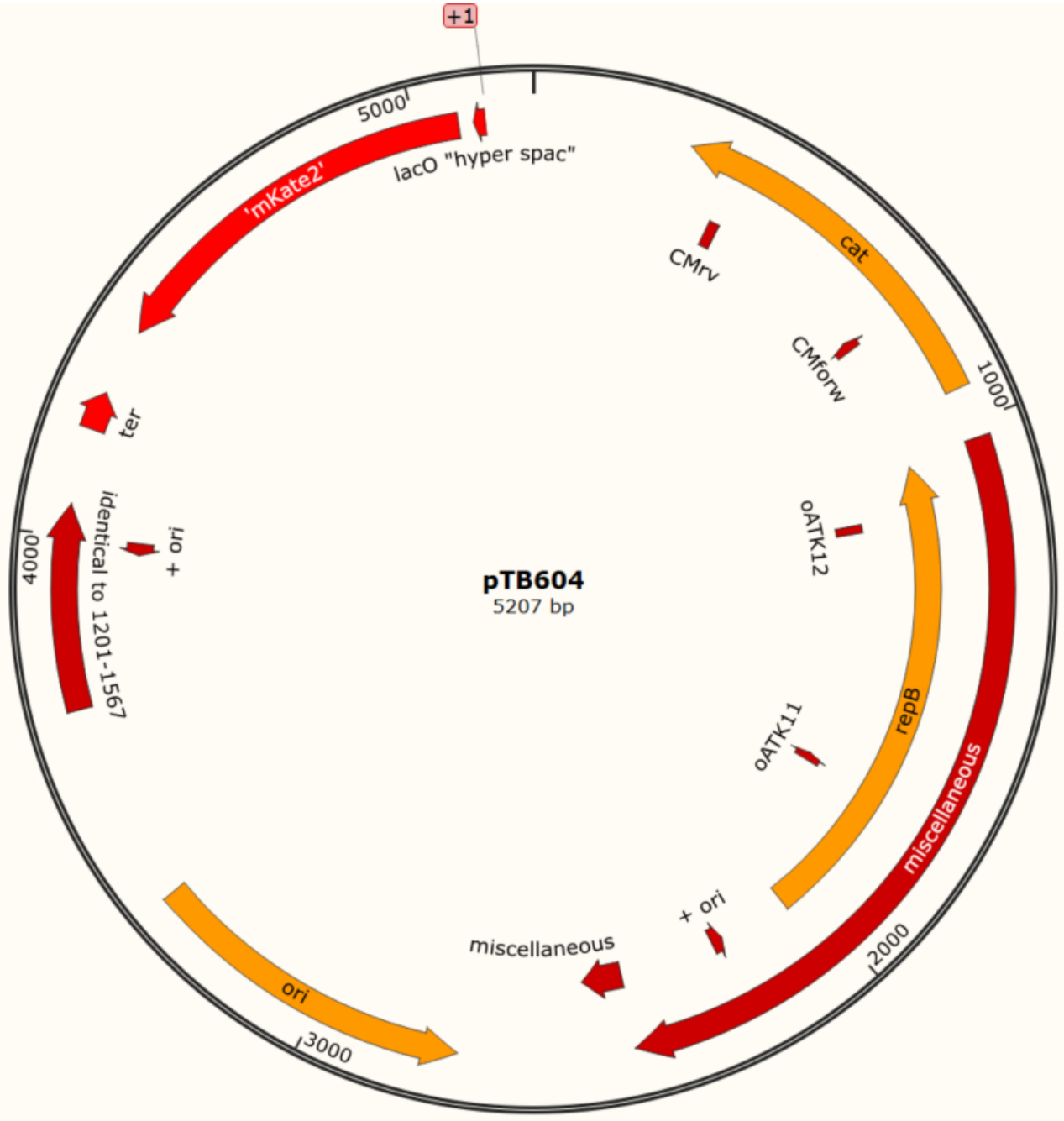
ori - origin of replication for *E. coli*,

repB - origin of replication for *Bacillus* species,

cat - chloramphenicol resistance,

gfp - GFP fluorescence protein,

lacO “hyper spac”- universal promoter.



Appendices 7. Plasmid pTB604 sequence and characteristic:

ori - origin of replication for *E. coli*,

repB - origin of replication for *Bacillus* species,

cat - chloramphenicol resistance,

'mKATE'- mKATE fluorescence protein,

lacO "hyper spac"- universal promoter.

STATEMENT OF AUTHORSHIP

This is to certify, that the intellectual content of this Thesis is the product of my own independent and original work and that all the sources used in preparing this thesis have been duly acknowledged.

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Tomislav Vološen