Charles University in Prague
Faculty of Pharmacy in Hradec Kralove
Department of Pharmacology and Toxicology

DIPLOMA THESIS

2017 Maria Olenic

Charles University in Prague

Faculty of Pharmacy in Hradec Kralove

Department of Pharmacology and Toxicology

Development of CRISPR-Cas9 based technology for genetic modification of *Lactococcus lactis subsp. cremoris*

Diploma thesis

Supervisors: Doc. Dr. Aleš Berlec

Prof. Dr. Borut Štrukelj

Prof. PharmDr. Petr Pávek

AUTHOR'S DECLARATION

I declare that this thesis is my original copyright work. Literature, materials and other resources that I used for this thesis are listed and properly cited in the relevant sections. This work was not submitted for obtaining a different academic degree.

Date: …… Signature: ……

ACKNOWLEDGMENTS

I am thankful for many people who helped me on my way of writing this thesis. Primarily I would like to say a great thank you to my main supervisor Assoc. Prof. Aleš Berlec, who is the head of the project I have been working on. His patience, involvement and ideas were crucial for making my experimental work possible. Also I am grateful to MPharm. Katja Škrlec and other people who worked with me and helped to gain all necessary skills for research in biotechnology department of Jožef Stefan Institute, Ljubljana.

I would also like to say thank you to Prof. Borut Štrukelj who is working in University of Ljubljana and offered me to join the project in Jožef Stefan Institute and to Assoc. Prof. Přemysl Mladěnka who connected me with Prof. Borut Štrukelj in term of Erasmus + program.

Also I am thankful to my supervisor in Faculy of Pharmacy in Hradec Králové Prof. Petr Pávek who helped me to write the thesis and reviewed it.

For the graphical support I would like to say a big thank you to Bc. Jozef Hašák who drew many of the schemes in this work.

Last but not least I am saying thank you to all of my family members and my friends who supported and motivated me on my way to finishing this thesis.

ABSTRACT

Development of CRISPR-Cas9 based technology for genetic modification of *Lactococcus lactis subsp. cremoris*

Lactococcus lactis, a bacterium from the group of Lactic Acid Bacteria (LAB), is a widely used bacterium and has a property of lactic acid production from lactose. It is an important microorganism used in fermentation of cheese products, but it also became the first genetically modified microorganism used alive for therapeutic reasons. LAB are also common probiotics taken as a supplement in mild diarrhea. The aim of this study is to develop technology that allows to modify *Lactococcus lactis* using Clustered Regularly Interspaced Palindromic Repeats-Cas9 system, that will become faster, easier and relatively cheap tool for genetic engineering of this bacterium.

First part of the project is designed to test cells containing two plasmids, and how efficiently Cas9 expressed from one plasmid is cutting a targeted gene on another plasmid (also called plasmid curing). For this I implemented the erythromycin resistance gene and designed CRISPR-Cas9 system aimed to disable this gene and measured activity of Cas9 protein by growing cells with designed plasmids in medium with or without erythromycin and comparing their optical density.

The second part of the project was based on genetic modification of cell's chromosome using homologous recombination and applying CRISPR-Cas9 as a tool for eliminating cells which remained unchanged.

For both experiments I was using NIsin Controlled gene Expression system (Mo Bi Tech, 2008). Plasmid genes expression was induced by introduction of nisin into the growth medium.

Experiments showed some promising results although the genetic design of plasmids and the protocols of cell growth still require some changes and adjustments. The project was developed by the team of scientists in the laboratory of biotechnology in Jožef Stefan's Institute, Ljubljana, Slovenia (Berlec et al., 2017).

ABSTRAKT

Vývoj na CRISPR-Cas9 technologie pro genetickou modifikaci *Lactococcus lactis subsp. cremoris*

Lactococcus lactis, také známý jako bakterie kyseliny mléčné (LAB), je široce používaná bakterie produkující kyselinu mléčnou z laktózy. Jedná se o důležitý mikroorganismus používaný při fermentaci sýrových produktů, stal se ale také prvním geneticky modifikovaným mikroorganismem, který byl použit z terapeutických důvodů. LAB je také běžné probiotikum užívané jako doplněk stravy při mírném průjmu. Cílem této studie je vyvinout technologii, která umožňuje modifikovat *Lactococcus lactis* pomocí systému Clustered Regularly Interspased Palindromic Repeats - Cas9 (CRISPR-Cas9), který se stane rychlejším, jednodušším a poměrně levným nástrojem pro genetické inženýrství této bakterie.

První část projektu je testoval efekt dvou plazmidů, jeden plazmid obsahoval rezistenci na antibiotikum erytromycin, druhý plazmid systém CRISPR-Cas9, který rezistenci deaktivoval. Selekcí v médiích s antibiotikem a sledováním optické hustoty baktérií byla potvrzena účinnost tohoto systému.

Druhá část projektu byla založena na genetické modifikaci buněčného chromozomu za použití homologní rekombinace a poté byla použita CRISPR-Cas9 jako nástroj pro eliminaci buněk, které zůstaly nezměněné.

Pro oba experimenty jsem použil NIsin genový expresní systém (Mo Bi Tech, 2008). Exprese plazmidových genů byla indukována zavedením nisinu do růstového média. Experimenty ukázaly některé slibné výsledky, i když genetický návrh plazmidů a protokoly růstu buněk stále vyžadují určité optimalizace a úpravy.

Projekt byl realizován ve spolupráci s kolegy z biotechnologické laboratoře v Ústavu Jožefa Štefana, Lublaň, Slovinsko (Berlec a kol., 2017).

Abbreviations

bp – base pairs

CFU – Colony Forming Units

CRISPR – Clustered Regularly Interspased Short Palindromic Repeats

GRAS – Generally Recognized As Safe

IL-10 – interleukin 10.

LAB – Lactic Acid Bacteria

LL or *L.lactis* – *Lactococcus lactis*

MCS – Multiple Cloning Site

NHEJ – Non-Homologous End Joining

NICE – Nisin Controlled Expression system

OD_{600} – Optical density measured at the wavelength 600 nm

PCR – Polymerase Chain Reaction

RBS – Ribosome Binding Site

sgRNA – single guide RNA

Table of Contents

1. Introduction

Inflammatory bowel diseases are widespread chronic conditions which are still hardly manageable and lack an effective safe treatment. Currently used anti-inflammatory agents, either glucocorticoids or non-steroid anti-inflammatory agents, have unpleasant generalized side effects and are not optimal for long term recovery. Researchers nowadays are trying to develop new approaches in management of these diseases.

Some probiotics are known to relieve the symptoms in antibiotics-induced mild diarrhea (Guandalini, 2011), but are not suitable for more severe conditions. Our idea is to enable the genetic programming of certain bacterial strains to deliver and express recombinant immunomodulatory proteins, locally, in the intestine. An example is the delivery of anti-inflammatory cytokine IL-10, that showed some positive results treating colitis in mice (Steidler et al., 2000). Means for up and down-regulation can also be developed in order to command bacteria when to launch the expression.

This approach faces several hurdles. First of all, although gene engineering technologies for bacteria exist, they are usually time consuming and expensive. After highly efficient CRISPR-Cas9 technology was described, vast majority of the research was focused on eukaryotes with a little attention to prokaryotes (Choi and Lee, 2016). To develop drug-delivering probiotics we need to create an efficient CRISPR-Cas9 based editing tool for the bacteria of choice. In this project I focus on *Lactococcus lactis* subsp. *cremoris* and on the technique which will become optimal for this microorganism.

2. Aims

The final goal of the project is to develop a single plasmid CRISPR-Cas9 based tool and a protocol for fast and effective genetic modification of *Lactococcus lactis* that will enable the isolation of transgenic strain within weeks. This will allow other researchers to create different strains of bacteria fast, and possibly to find the one that will help to fight inflammatory conditions in the bowel.

In the experiment of chromosome modification we have chosen HtrA housekeeping protease gene to be modified (Poquet I. et al, 2000). The desired transgenic form of this gene results in dysfunctional protease production. This is not lethal to the cells because it is just one of the proteins present on the outer surface of the cell membrane and that helps with protein digestion. But this mutation is preferable for cells that will express recombinant anti-inflammatory proteins because it will eliminate possible auto-proteolysis of the secreted product.

3. Theoretical part

3.1. Lactococcus lactis subsp. cremoris

This microorganism belongs to group of bacteria also known as Lactic Acid Bacteria. They are Gram-positive, nonsporulating, non-pathogenic bacteria that group in pairs or short chains. *L.lactis* belongs to the group of microorganisms generally recognized as safe (GRAS). Their characteristic feature is utilizing lactose for ATP production and secreting lactic acid as a byproduct in this process. LAB genome is known to be low in GC content (guanine-cytosine nucleotides), which means it is less stable during denaturation.

L.lactis is widely used in the process of fermentation of cheese and other milk products. Subsp. *cremoris* also has industrial importance in this field (Laroute et al., 2017).

Lactococcus lactis was the first transgenic living microorganism used in humans for therapeutic reasons (1). It has shown a potential is the treatment of inflammatory bowel diseases.

In the project I am working with commercially available host strain named *Lactococcus lactis* NZ9000 (© MoBiTec GmbH, 2008). This strain already contains nisin controlled transduction system (genes *nisRK*) integrated in its chromosome (*pepN* gene).

3.1.1. Storage conditions

Labeled Eppendorf tubes with 50 or 100µl of cells are stored at -70ºC. Before using are defrosted on ice.

3.1.2. Growth medium

GM17 liquid medium

Composition: M17 broth acc. to TERZAGHI, purified water, glucose.

Protocol: 42.5 g of M17 broth is dissolved in 1L of purified water, autoclaved and then 6.25 g of sterile glucose sirup (40%) is added to the mixture under aseptic conditions in laminar box. Antibiotics may be added to the medium if plasmid selection pressure is necessary.

GM17 agar plates

Same as GM17 liquid medium with the addition of 15g of agar to 1L of medium before autoclaving. After autoclaving and addition of glucose the medium is poured to Petri dishes. After cooling the plates are stored in a cool environment.

SGM17 liquid medium

GM17 medium with 20 mM $MgCl_2$ and 2mM CaCl2. Used for growth of newly transformed cells.

3.1.3. Competent cells

Competence is the ability of cells to uptake an extracellular DNA via the process of transformation (Natural competence, Wikipedia). In order for the plasmid to come across the bacterial membrane I needed to prepare eclectically competent *L.lactis* with slightly destabilized membrane that will open up the pores during application of electric pulse. The protocol can be found in the paper of NICE® Expression System for *Lactococcus lactis* (Mo Bi Tech, 2008).

3.1.4. Transformation of *L.lactis*

Transformation is a process of delivering plasmid inside the cell's cytoplasm. For *Lactococcus lactis* I was using electroporation method under specified conditions. Normally 48µl of competent cells are mixed with 2µl of the isolated plasmid and placed into the electroporation cuvette. The cuvette is placed in a Biorad Genepulser® II to perform the electroporation with electric potential 2000 V, capacitance 25 µF and resistance 200 Ω. The pulse duration is 4.5-5 msec. After that the cells are resuspended with up to 1ml of SGM17 medium and incubated 2 hours at 30 ºC and then various volumes can be inoculated to the agar plates (10, 20, 100, 200 µl or entire volume). The plates are left to grow 2 days at 30 ºC.

3.2. Plasmid construction

3.2.1. pNZ8148

pNZ8148 is a standard commercially available plasmid designed for replication and gene expression in a broad host range of Gram positive bacteria (Figure 1). The genes will be expressed are cloned at the multiple cloning site region (MCS) and their expression is induced by activation of nisin promoter (Pnis) located in front of MCS. The plasmid also contains chloramphenicol acetyltransferase gene (Cm) for antibiotic selection pressure during bacterial growth. Therefore only the cells that accepted the plasmid during transformation will be able to survive in the growth medium containing chloramphenicol (concentration 10 µg/ml). In the Figure 1 there is an original vector map.

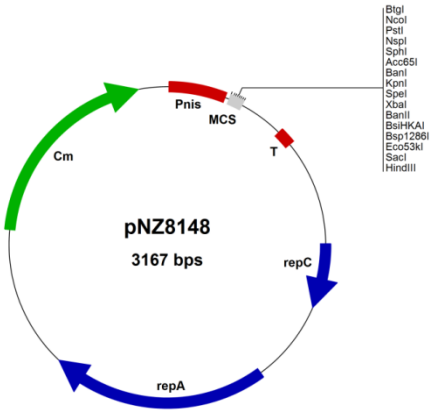


Figure 1. **Vector map of the plasmid pNZ8148.** (Mo Bi Tech, 2008)

Before I started to assemble CRISPR on the plasmid it was necessary to perform restriction site analysis to choose the right restriction enzymes for cloning. Long nucleotide sequences like Cas9 gene contain many restriction sites that are therefore not suitable for cloning. It is crucial to confirm that none of the sequences we are working with contain the restriction sites that will be used during cloning. For that we used RestrictionMapper on-line software.

After plasmid backbone and Cas9 gene sequences analysis we found that suitable restriction sites are: XbaI, XhoI, PacI, HindIII, SalI. First four are present in the multiple cloning site (MCS) of the plasmid and will be used to insert our CRISPR genes.

Later, after several subsequent cloning steps I achieved different constructs of the pNZ plasmid that made my experiments possible. Both projects (plasmid curing and chromosome modification) incuded pNZ plasmid. Its design is similar to the certain point for both experiments. The major difference comes in sgRNA sequence because it has to be constructed separately for each gene that we need to cut.

3.2.1.1. Basic structure design

First of all the second nisin promoter was cloned and inserted after the first one using restriction sites XbaI and XhoI (Figure 2). Dual nisin induction system showed good results in the experiments of my colleagues (Berlec A. et al, 2017). Then the ribosome binding site (RBS) was removed from the end of the first promoter because I will place sgRNA gene after this promoter and sgRNA should not be translated to the protein after it has been transcribed from the plasmid.

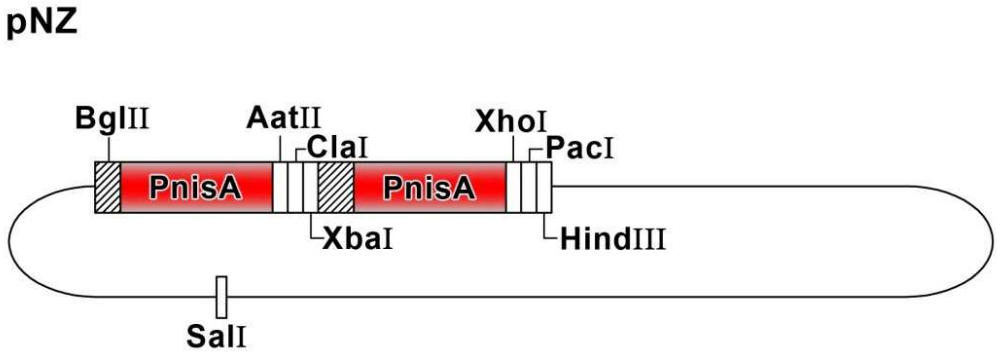


Figure 2. **pNZ plasmid with second nisin promoter PnisA cloned in MCS.**

CRISPR-Cas9 elememts now can be cloned in this plasmid. Firstly Cas9 gene of known sequence is cloned using XhoI and PacI sites. At this point we obtain the plasmid called pNZ-Cas9 which will be used for further construction of plasmids for different experiments.

Selected sgRNA (designed for specific purpose; see 3.3.1.) is inserted to the plasmid using AatII and XbaI restriction enzyme sites (Figure 3). This allows targeting Cas9 nuclease activity to selected sequences.

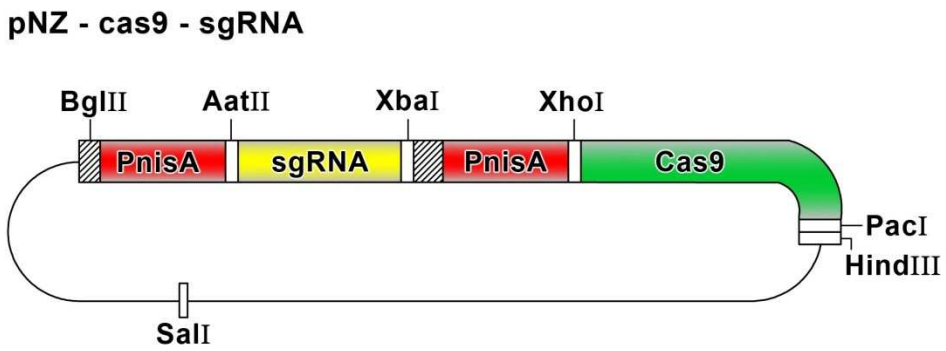


Figure 3. **Basic pNZ plasmid construct design used for targeted gene knock out**. SgRNA gene expresses RNA fragment that plays a role in site recognition and Cas9 gene expresses the nuclease that cleaves that site.

3.2.1.2. Construction of pNZ-Cas9-sgErm

pNZ-Cas9-sgErm is used in the plasmid curing experiment (see 4.1.). The design of this plasmid resembles the basic structure described above with the specification of sgRNA. Single guide RNA transcribed from this plasmid aligns to erythromycin resistance gene and is named sgErm. Function of the whole plasmid is to disable another plasmid (pMSP or pIAV) by cutting the erythromycin resistance gene in its sequence.

3.2.1.3. Construction of pNZ-Cas9D10A-sgHtra-HtrAD

Function of this plasmid is complex because its activity is necessary for bacterial chromosome modification.

CRISPR-Cas9 system is coding single guide RNA targets the nucleotide sequence of the wild-type protease HtrA (sgHtrA)., HtrA gene is absent from the transgenic type, thus targeting it with Cas9 eliminates only the unmodified cells. Also instead of Cas9 nuclease I cloned Cas9D10A nickase to the CRISPR system following the paper on *Clostridium cellulolyticum* (see 3.3.2.).

Besides CRISPR elements the plasmid also contains homologous regions of the HtrA protease called HtrAD fragment (Figure 4). This fragment enables homologous recombination with the cell's chromosome - and inactivation of the htrA gene.

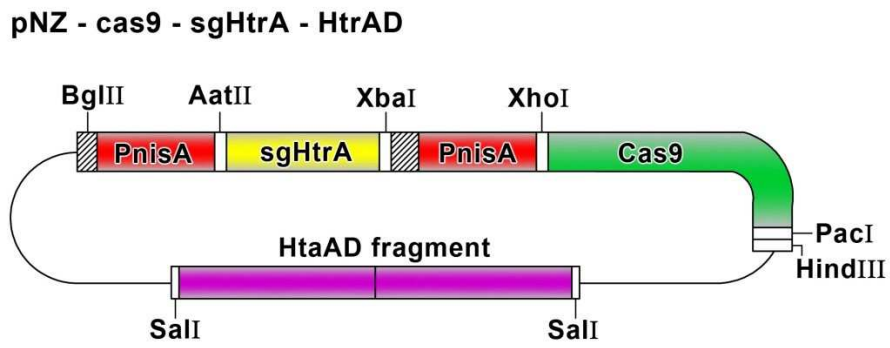


Figure 4. **pNZ-Cas9-sgHtrA-HtrAD plasmid construct that contains homologous regions of the HtrA protease gene (HtrAD) and CRISPR-Cas9 elements targeting wild-type serine protease.**

To clone HtrAD fragment inside the plasmid I first amplified it directly from the bacterial chromosome using Taq PCR protocol. Knowing the nucleotide sequence of the wild-type protease we designed primers (Htr-A-Sal, Htr-B-Bam, Htr-C-Bam, Htr-D-Sal) that anneal and amplify two fragments from the original gene (Figure 5). These fragments were isolated, treated with restriction enzyme BamHI, and ligated into HtrAD fragment.

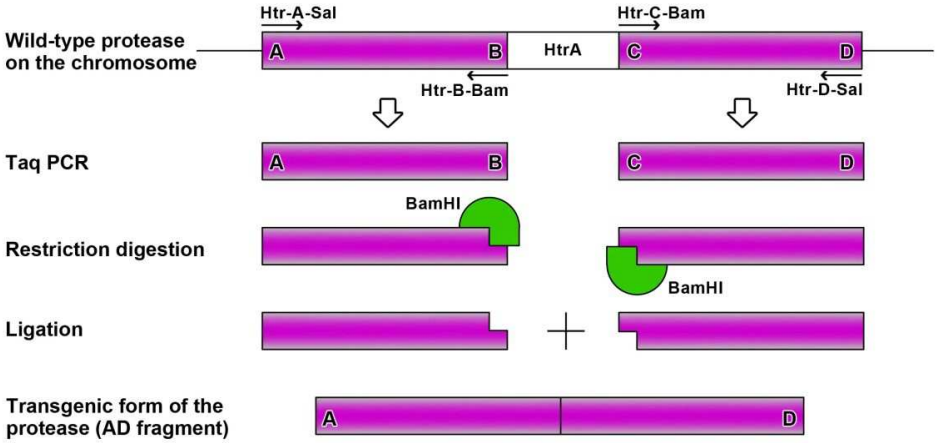


Figure 5. **HtrAD fragment construction process.**

3.2.1.4. Plasmid genes expression regulation system

In my experiments I was using NICE® system. To induce the transcription of CRISPR elements from the pNZ plasmid it is necessary to add nisin in concentration 25 ng/ml to the growth medium. Nisin is an anti-microbial peptide, made out of 34 amino acids, that in sub-inhibitory concentrations (0.1-5 ng/ml) induces expression of the gene under the control of nisin promoter (Mo Bi Tech, 2008).The strain of bacterium which I am using already codes all necessary signal transduction components (genes *nisRK*) in its chromosome. After the activated complex binds the nisin promoter (Pnis) on the plasmid the transcription begins (Figure 6).

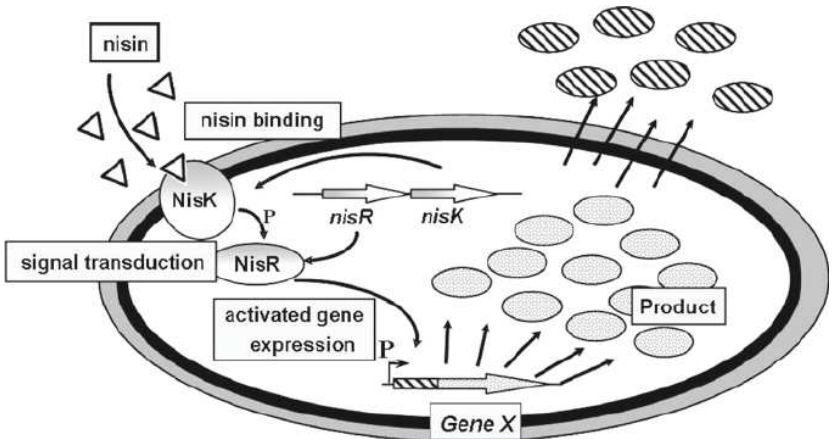


Figure 6. **The principle of nisin controlled expression system inside bacteria** (Mo Bi Tech, 2008).

3.2.2. pMSP3535

pMSP3535 (Bryan et al., 2000), also called pMSP-IRFP or pMSP in my work, is a plasmid used in our experiment of plasmid curing. The relevant components are the erythromycin resistance gene, nisin promoter and a reporter gene of Infra Red Fluorescent Protein (IRFP). The theoretical advantages of this plasmid are that it is inducible and the level of protein expression from this plasmid can be precisely measured by monitoring of fluorescence. Complete sequence can be found at GenBank: AY303239.1.

3.2.3. pIAV7

pIAV7 is a broad range vector for Lactic Acid Bacteria that contains erythromycin resistance gene (Pérez-Arellano et al., 2001). Its expression is not controlled by nisin and it does not contain a reporter gene that can help to quantify the expression rate. pIAV7 was used as a second choice plasmid in the plasmid curing test due to unsuccessful experiment with pMSP plasmid.

3.3. CRISPR-Cas9 array design

CRISPR-Cas9 system is composed of three functional parts that enable bacterial adaptive immunity of some strains: CRISPR RNAs (crRNA), tracrRNA and Cas9 protein. This system belongs to type II, class 2 CRISPR-Cas systems, which means it only utilizes a single effector protein to perform site specific cleavage (Chylinski et al., 2014). This type of CRISPR-Cas system was first described in Steptococcus pyogenes.

Wild type CRISPR-Cas9 system also requires RNase III to be able to transcribe RNA elements. Simpler approach was designed at the University of California by creating a single guide RNA (sgRNA) which is a chimeric RNA made out of two parts: crRNA and tracrRNA (Jinek et al., 2012). This approach allows faster and easier plasmid design therefore I was using it in this research.

There are various types of Cas proteins available for genetic engineering. In my experiments I was testing Cas9 which is a nuclease and creates double strand blunt-ended breaks in the DNA and Cas9D10A which is a nickase and makes a single strand break. The only difference between these two proteins is an amino acid alanine on the position 10 present in the latter. The decision to test Cas9 nickase on *L.lactis* was made based on successful experiments with *Clostridium cellulolyticum* which showed that the use of wild-type Cas9 is too lethal for the bacteria which lack NHEJ expression elements (Xu et al., 2015).

3.3.1. Single guide RNA design and cloning

The general sequence of sgRNA is known to be:

```
>sgRNA
NNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAAC
TTGAAAAAGTGGCACCGAGTCGGTGCTTT
```

The first twenty nucleotides (N) are designed specifically to find and anneal to the target gene, while the rest of the sequence is constant for all sgRNAs and is required to attach to Cas9 protein. To find an appropriate sequence it is necessary to analyze the gene we want to cleave.

3.3.1.1. SgErm

In the first experiment I tested the CRISPR-Cas9 system in the bacteria that contain a plasmid with erythromycin resistance gene to see how well can the Cas9 protein cut and disable this gene. First twenty nucleotides of sgRNA against erythromycin resistance gene (sgErm) should be taken from the sequence of this gene. The choice is not random. Sequence should be located just in front of a protospacer adjacent motif (PAM) which is typically made out of three nucleotides NGG or NCC. PAM region is recognized by one of the crRNA complexes that enables the annealing of guide RNA and subsequent cleavage of the gene by the Cas9 protein. It is important that PAM region is not present in sgRNA, otherwise the plasmid coding this sgRNA will be cleaved too.

This is the sequence of erythromycin resistance gene (GenBank: AY303239.1) with highlighted PAM regions:

>ErmR
ATGAACAAAAATATAAAATATTCTCAAAACTTTTTAACGAGTGAAAAAGTACTCAACCAAATAATAAAACAATTGAA
TTTAAAAGAAACCGATACCGTTTACGAAATTGGAACAGGTAAAGGGCATTTAACGACGAAACTGGCTAAAATAAGTA
AACAGGTAACGTCTATTGAATTAGACAGTCATCTATTCAACTTATCGTCAGAAAAATTAAAACTGAATACTCGTGTC
ACTTTAATTCACCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAGTATTCCTTA
CCATTTAAGCACACAAATTATTAAAAAAGTGGTTTTTGAAAGCCATGCGTCTGACATCTATCTGATTGTTGAAGAAG
GATTCTACAAGCGTACCTTGGATATTCACCGAACACTAGGGTTGCTCTTGCACACTCAAGTCTCGATTCAGCAATTG
CTTAAGCTGCCAGCGGAATGCTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCAC
AGATGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGAATATCGTCAACTGT
TTACTAAAAATCAGTTTCATCAAGCAATGAAACACGCCAAAGTAAACAATTTAAGTACCGTTACTTATGAGCAAGTA
TTGTCTATTTTTAATAGTTATCTATTATTTAACGGGAGGAAATAA

A lot of PAM regions can be found in the erythromycin resistance gene. The best fragment for guide RNA can be chosen by following guidelines, including analysis of GC content (Jacob Corn). By following them we have chosen the sequence: TTGGATATTCACCGAACACT. Eventually, the sequence of sgRNA designed to target erythromycin resistance gene (named sgErm) was as follows:

>sgErm

TTGGATATTCACCGAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTT
GAAAAAGTGGCACCGAGTCGGTGCTTT

To create this sequence for further cloning our team decided to perform alignment and amplification of two overhanging primers (Figure 7) using Taq PCR protocol. SgRNA gene is not very long, so the idea was to design and order two primers – forward (F) and reverse (R) from Integrated DNA Technologies®. The sequences are relatively long for usual primers used in PCR, but the procedure was successful and we were able to isolate the correct gene of sgErm. The sequences of the primers are (forward and reverse):

>sg-erm3-F
GACGTCTTGGATATTCACCGAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATC

>sg-erm3-R
GCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTTTTCTAGA
TCTAGAAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTTAACTTGC

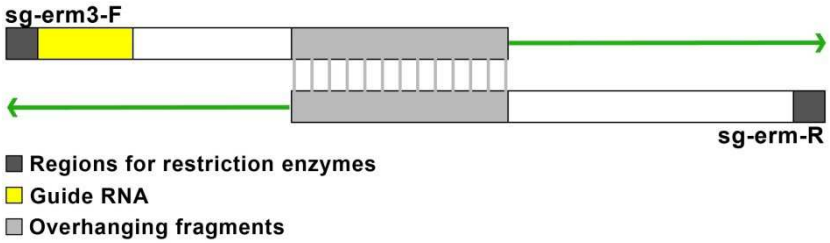


Figure 7. **SgErm synthesis principle using amplification of two overlapping primers sg-erm3-F and sg-erm-R in Taq PCR.**

3.3.1.2. SgHtrA

In the experiment where I am changing a protease gene inside bacterial chromosome (see 4.2.) I use a different guide RNA. This time we want to inactivate the bacteria with a wild-type, unchanged protease, therefore sgRNA is designed to anneal to the fragment which is only present in a wild-type protease (Figure 8). The fragment is called HtrA and has the following sequence:

```
>HtrA
AAAGACGTGGCAACTTTCGCTGATTCAAGCAAGTTAACTATTGGTGAACCAGCTATTGCAGTCGGCTCACCTTTAGG
TAGCCAGTTTGCTAATACTGCAACCGAAGGAATTCTGTCTGCAACAAGTCGTCAAGTCACTTTGACTCAAGAAAATG
GTCAAACAACAAGTATCAATGCGATTCAAACGGATGCTGCCATTAACCCTGGTAACTCAGGTGGAGC
```

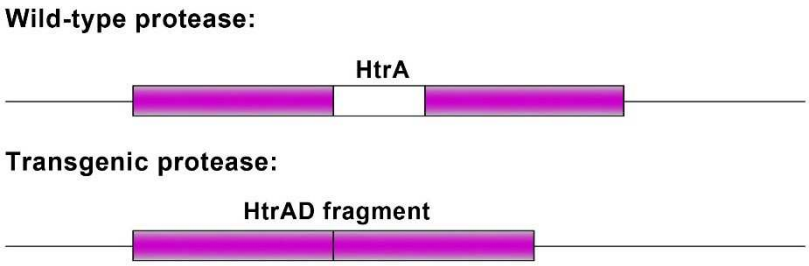


Figure 8. **Difference between wild-type and inactivated type of serine protease in *L.lactis***

After PAM regions analysis a suitable guide RNA has been chosen: ACGGATGCTGCCATTAACCC. Therefore the final sequence of sgHtrA is:

```
>sgRNA_HtrA
ACGGATGCTGCCATTAACCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAA
AGTGGCACCGAGTCGGTGCTTT
```

Now to synthesize and clone this sequence inside pNZ plasmid I used sgErm gene, that we isolated before, and amplified it using Taq PCR with a different forward primer (sg-Htra-F). The resulted PCR product will be a sgRNA with a different guide region (Figure 9).

Forvard and reverse primers:

>sg_HtrA_F
GACGTCACGGATGCTGCCATTAACCCGTTTTAGAGCTAGAAATAGCAAG
>sgRNA-R
TCTAGAAAAGCACCGACTCG

Template (regions where primers align are underlined):

>sgErm

TTGGATATTCACCGAACACTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAA
AGTGGCACCGAGTCGGTGCTTT

PCR product: sgHtrA with restriction sites:

>sgRNA_HtrA

GACGTCACGGATGCTGCCATTAACCCGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACT
TGAAAAAGTGGCACCGAGTCGGTGCTTT

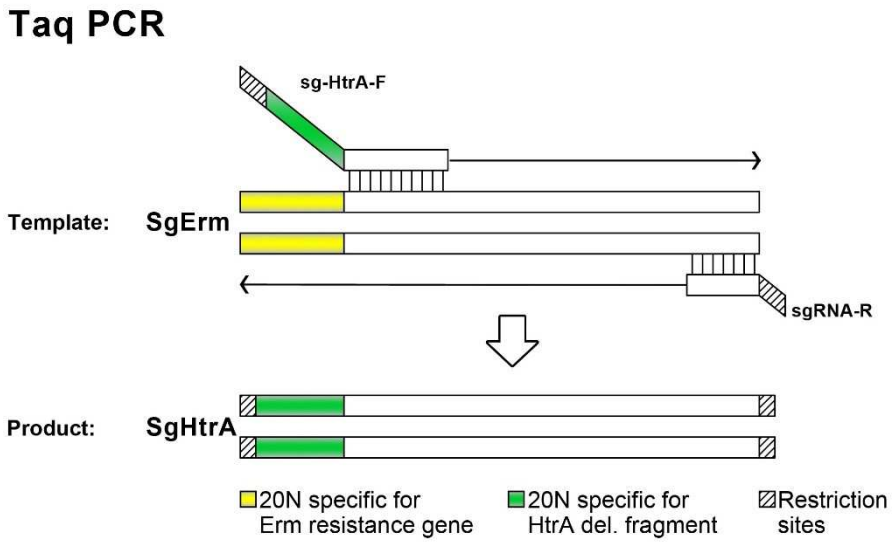


Figure 9. **Principle of sgHtrA gene synthesis using Taq PCR, sgErm as a template and primers sg-HtrA-F and sgHtrA.**

3.3.2. Cas9 and Cas9D10A comparison

Cas9 is a nuclease which creates double strand breaks in DNA. The experiments on *Clostridium cellulolyticum* showed that implementation of Cas9 was too lethal for these bacteria (Xu T et al., 2015) and Cas9 nickase was applied instead. Cas9D10A is a modified version of Cas9 protein with a single nucleotide mutation at the 10^{th} codon position: instead of asparagin (D, gat) it codes alanin (A, gct). In contrast with Cas9, Cas9D10A is a nickase, which means it only creates a single strand break and should not be as lethal for cells.

In my experiments the intention was to create two equivalent pNZ plasmids with different versions of Cas9 protein to compare their function inside *Lactococcus lactis*. But unfortunately, due to experimental issues, I was not able to clone the full construct of final plasmid with Cas9 protein: pNZ-Cas9-sgHtrA-HtrAD. I was only testing the plasmid with the nickase: pNZ-Cas9D10A-sgHtrA-HtrAD. Therefore the comparison was not possible.

4. Experimental part

4.1. Plasmid curing

4.1.1. Principle and aims

This experiment is designed to find out if CRISPR-Cas9 system is generally able to function inside *Lactococcus lactis*, meaning that all parts of CRISPR array are expressed from a plasmid under specified conditions and perform a desired function. The principle is based on gene expression tests of the bacteria containing different plasmids (Figure 10).

For that I transformed cells with two plasmids: one containing erythromycin resistance gene (pMSP or pIAV7) and, another, – CRISPR system (pNZ). Single guide RNA is designed to allign with erythromycin resistance gene (see 3.3.1.1.). Therefore the induced pNZ plasmid should express CRISPR-Cas9 system and Cas9 protein will cut the erythromycin resistance gene on the second plasmid that will make the cell sensitive to erythromycin again. This can be measured by growing bacteria in a medium with or without erythromycin (concentration 10 µg/ml; E10) and comparing optical density of both liquids (OD_{600}). Medium with the cells containing fully assembled CRISPR array (pNZ-Cas9-sgErm) and induced with nisin should show smaller OD_{600} in the presence of erythromycin than in its absence. Basically, erythromycin should become lethal to bacteria as they lose the resistance. That would be the evidence of Cas9 activity. As a control I took the cells transformed with the plasmid lacking sgErm in the construct (pNZ-Cas9). The control should not show a significant difference in growth in presence or absence of erythromycin since Cas9 nuclease is unable to find the target.

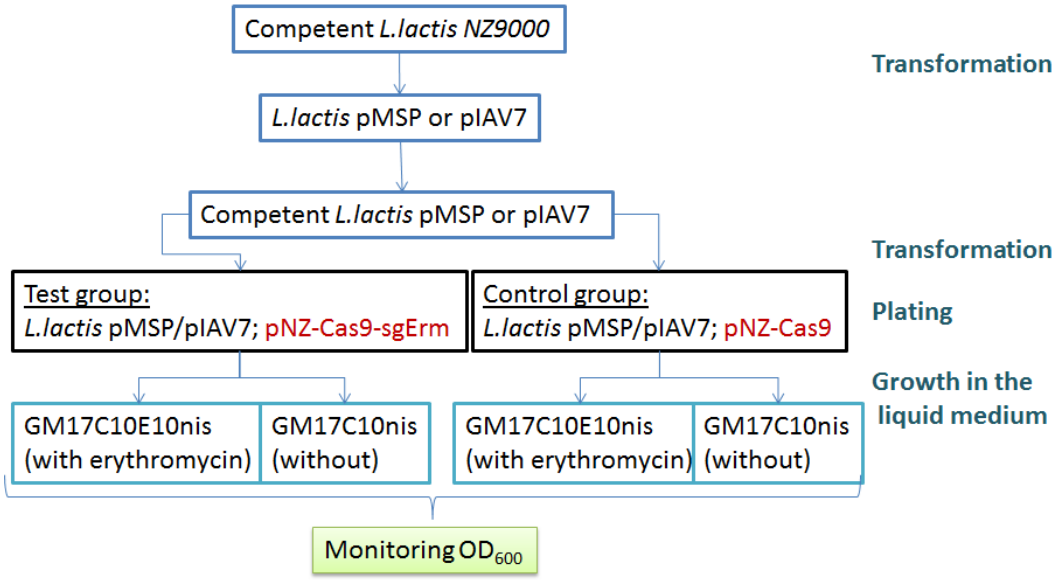


Figure 10. **General steps of the pMSP and pNZ replication test in *L.lactis*.** GM17 is a basic growth medium for *L.lactis*, C10 – chloramphenicol in concentration 10 µg/ml. E10 – erythromycin in concentration 10 µg/ml. Nis – nisin in concentration 25 ng/ml.

4.1.2. Experiment with pMSP-IRFP

Initially the plasmid with erythromycin resistance gene was chosen to be pMSP-IRFP (Bryan et al., 2000) It contains nisin promoter and a reporter gene of Infra Red Fluorescent Protein (IRFP) which will emit a different level of fluorescence at the different rate of expression.

The protocol of the experiment was based on the general principle described in the Figure 11. Nisin induction was performed in two ways: in the beginning of the growth phase or after cell cultures reached OD_{600} from 0.8 to 1.0. Fluorescence of samples was measured in Tecan Infinite® M1000 Pro Microplate Reader.

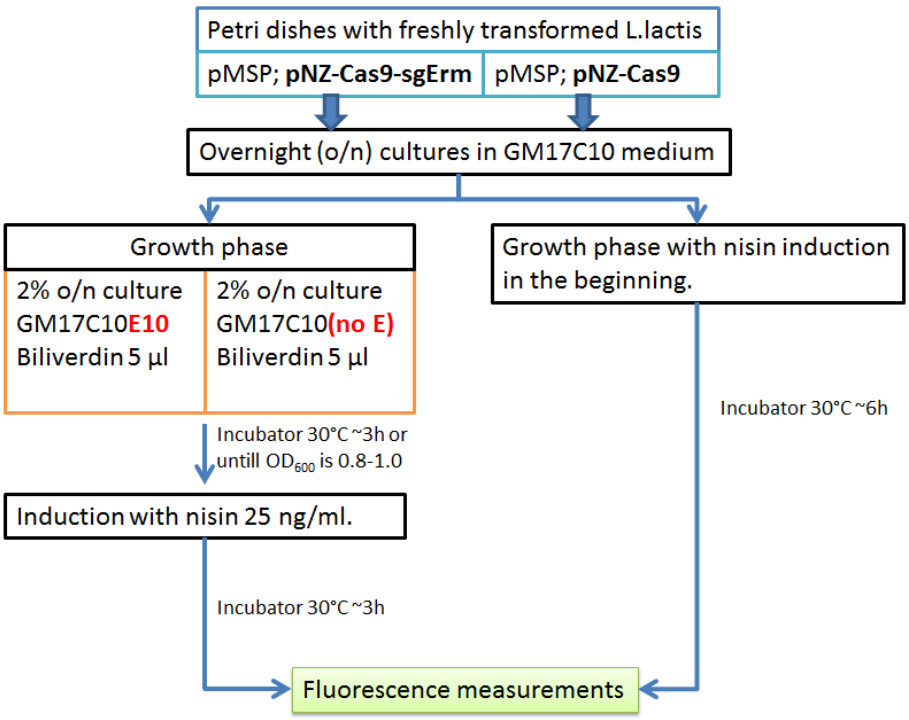


Figure 11. **Protocol of pMSP and pNZ plasmids replication test in *L.lactis*.** GM17 is a basic growth medium for *L.lactis*, C10 – chloramphenicol in concentration 10 µg/ml. E10 – erythromycin in concentration 10 µg/ml. Nis – nisin in concentration 25 ng/ml.

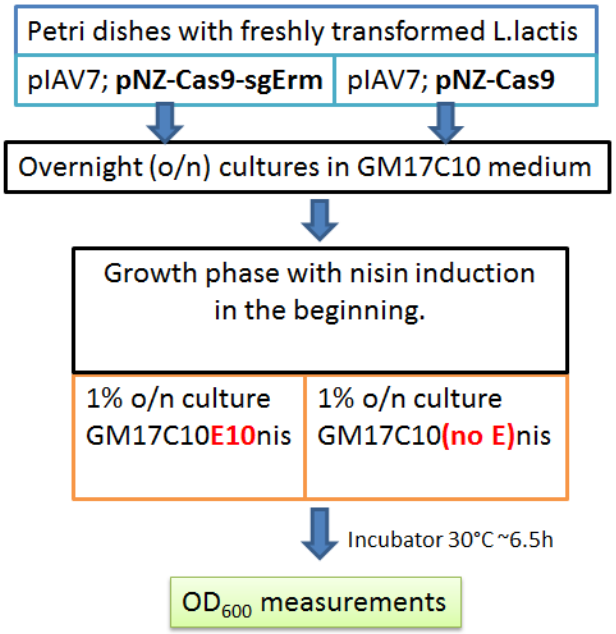
Unfortunately, the experiment was stopped because the results were non-reliable. Bacterial growth was slow and fluorescence was not intensive enough to notice the difference between samples and make any conclusions about CRISPR-Cas9 activity. Perhaps nisin induction does not work well when it is used with two different plasmids simultaneously in one cell. Therefore, I decided to quit this experiment and try to use another erythromycin resistance gene containing plasmid (pIAV7) for the same purpose.

4.1.3. Experiment with pIAV7

After unsuccessful experiment with pMSP I tried different plasmid which essentially contains the same erythromycin resistance gene, so I did not have to reconstruct sgErm. Since pIAV7 lacks IRFP I was not able to measure the fluorescence in the end of expression test. The only evidence of CRISPR activity was bacterial growth rate, which was measured through OD_{600}. If Cas9 protein is active and cuts pIAV7 plasmid, then the cells are losing their tolerance to erythromycin and it inhibits the growth of the culture, so theoretical OD_{600} should be lower.

4.1.3.1. Primary miniexpression test

At the beginning I did a primary expression test (Figure 12) on just one set of samples to find out if this approach works and got a positive result – OD_{600} of full CRISPR array in presence of erythromycin was the lowest.



LL pIAV7; pNZ-Cas9-sgErm		LL pIAV7, pNZ-Cas9	
E10	No E	E10	No E
0.242	0.378	0.396	0.427

Figure 12. **Protocol for primary expression test of pIAV7 and pNZ plasmids.**

Therefore I continued with a more statistically reliable expression test in Tecan Sunrise® absorbance microplate reader.

4.1.3.2. Tecan® miniexpression test

I loaded quadruplicates of samples with the different growth medium composition (Table 1) to the 96-well microplate:

LL transformed with plasmids:	Growth medium composition:
pIAV7; pNZ-Cas9-sgErm	GM17C10
	GM17C10E10
	GM17C10E10nis
pIAV7; pNZ-Cas9	GM17C10
	GM17C10E10
	GM17C10E10nis

Table 1. **Comparison of activity of two pNZ plasmid constructs: pNZ-Cas9-sgErm and pNZ-Cas9 (control construct).** Both constructs were examined by growing the transformed cells in different growth media listed in this table. GM17 is a basic growth medium for *L.lactis*, C10 – chloramphenicol in concentration 10 µg/ml. E10 – erythromycin in concentration 10 µg/ml. Nis – nisin in concentration 25 ng/ml.

Different growth medium composition allows the analysis of how bacterial growth rate is changing with addition of erythromycin and nisin.

The samples are heated to 30°C inside the microplate reader and the OD_{600} measurements are done every 20 seconds within one day. After the data was collected I plotted it into graph curves and compared the growth rates of different cultures. The results were reproducible in all four sets of samples therefore I have chosen one representative curve for each sample to compare with others.

In the Figure 13 there is a slight difference in the growth rate of cells with full CRISPR-Cas9 construct pNZ-Cas9-sgErm and partial pNZ-Cas9, although CRISPR is not lethal for *L. lactis* in presence of erythromycin. We also cannot judge the behavior of CRISPR-Cas9 system before we compare its activity in different medium.

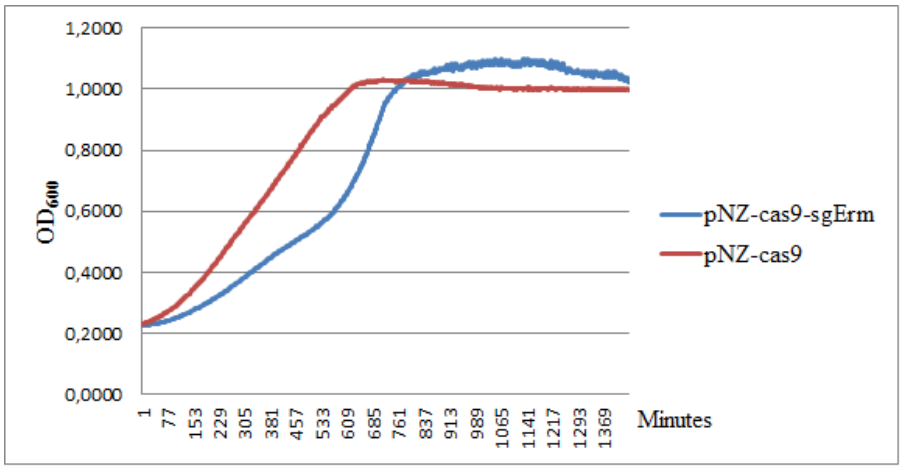


Figure 13. **Nisin induction of two different pNZ plasmid constructs expressed in transformed *L. lactis* grown in GM17C10E10 medium.** Growth rates are expressed in changes of optical density measured at 600 nm over the period of 24 hours.

On the figure 14 the same pattern of growth rates can be observed while growing bacteria with pNZ-Cas9-sgErm in the presence or absence of erythromycin. This similarity indicates that reduced growth rate is not a consequence of increasing erythromycin sensitivity and CRISPR-Cas9 affects cells in some other way. Therefore this experiment is not successful.

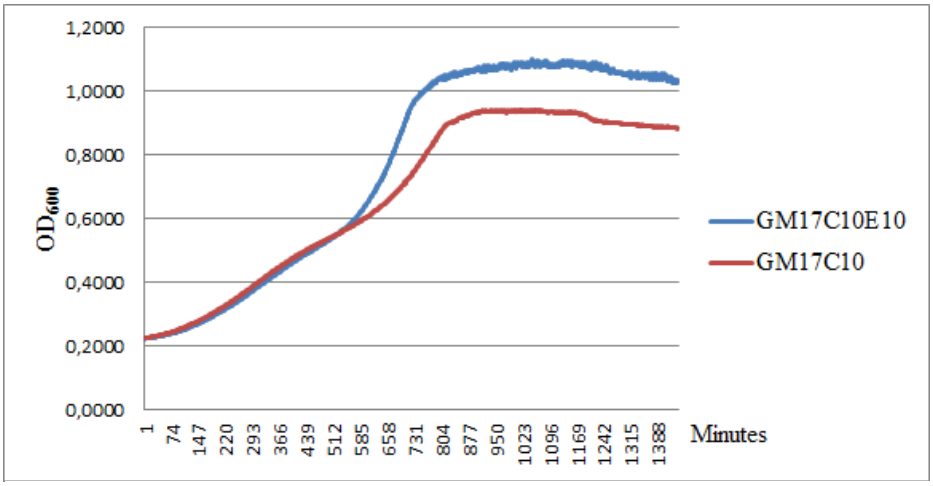


Figure 14**. Nisin induction of plasmid construct pNZ-cas9-sgErm expressed in *L. lactis* that were grown in two different growth mediums: GM17C10E10 (containing erythromycin) and GM17C10 (no erythromycin).** Growth rates are expressed in changes of optical density measured at 600 nm over the period of time of 24 hours.

As a result I proceeded to another protocol design which included multiple subculturing of the samples in different media and plating to the Petri dishes with different antibiotic concentrations.

4.1.3.3. Expression with multiple subculturing and plating

The principle of this expression test is based on three basic steps: growth phase, induction phase and growth on the solid medium. During development of the protocol I tried to optimize such parameters as: percentages of subculturing volumes (how much of the previous culture will be added to the next culture), concentrations of erythromycin and nisin, time of incubation, amount of cultures to be plated. First positive results were achieved by usin the protocol described in Figure 16. I was able to obtain significantly lower amount of CFU at the plate with *L. lactis* containing the full construct of CRISPR-Cas9. The first results were non-quantifiable due to colony overgrowth (Figure 17). Subsequently, the test was repeated with the only difference that at the end drop plate method was used to inoculate cultures to the agar.

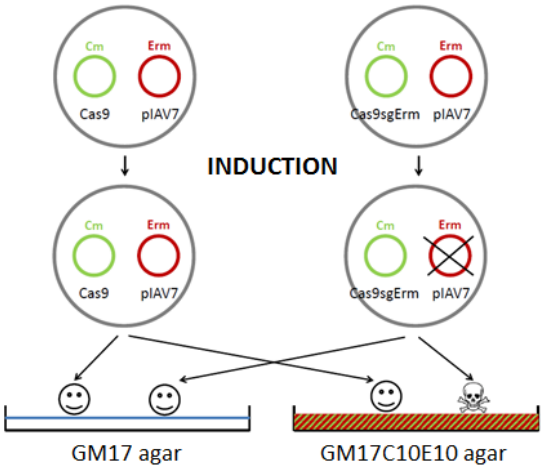


Figure 15. **Principle of the experiment with multiple subculturing and induction of transformed *L. lactis* (with pNZ and pIAV7 plasmids) and their subsequent plating to the solid medium with different composition.** After growth and induction, cells with fully constructed pNZ plasmid are not expected to grow on erythromycin containing plates.

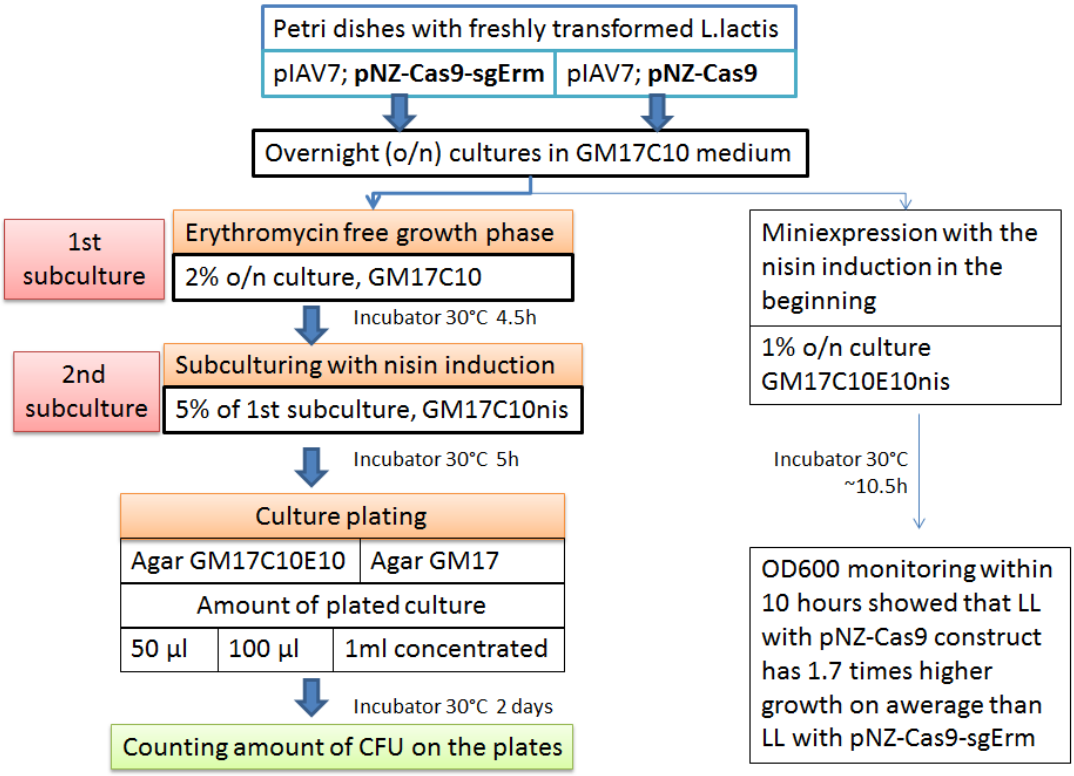


Figure 16. **Protocol for testing of pNZ-Cas9-sgErm and pIAV7 plasmids in *L.lactis* that includes multiple subculturing and plating steps.** GM17 is a basic growth medium for *L.lactis*, C10 – chloramphenicol in concentration 10 µg/ml. E10 – erythromycin in concentration 10 µg/ml. Nis – nisin in concentration 25 ng/ml.

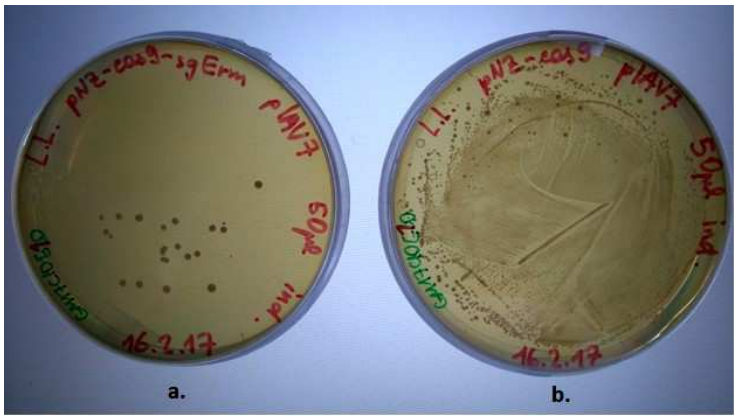


Figure 17. **a. *L.lactis* colonies transformed with pNZ-Cas9-sgErm and pIAV7, induced with nisin and plated to the GM17C10E10 agar.**

b. *L.lactis* colonies transformed with pNZ-Cas9 and pIAV7, induced with nisin and plated to the GM17C10E10 agar. These colonies are overgrown.

To reproduce the results and make them more reliable the protocol was repeated (Figure 15); however, drop plating method was used in the last step. ANOVA statistical test was used to evaluate the data. The results are graphically demonstrated on the graph (Figure 18).

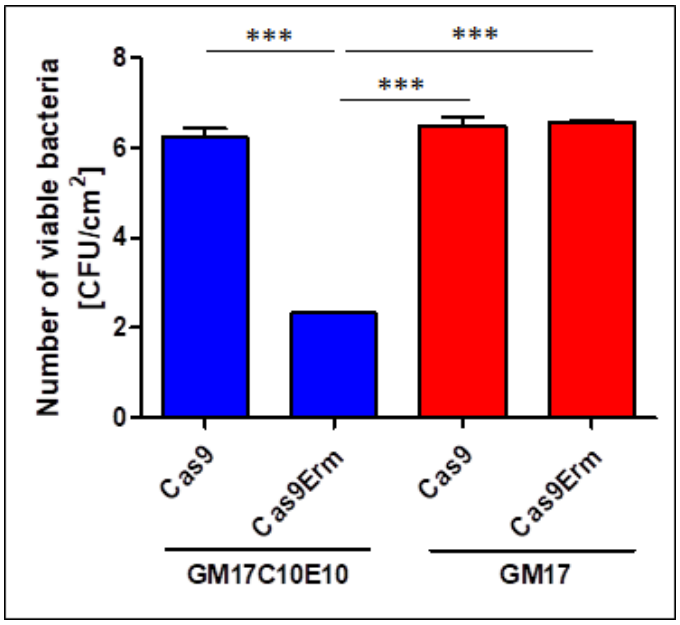


Figure 18. **Statistical evaluation of CFU obtained from drop plating method of *L. lactis* containing pNZ plasmid construct with or without sgErm gene and on different agar medium:** GM17C10E10 – with chloramphenicol and erythromycin; GM17 – without any antibiotics.

4.1.4. Evaluation of results

To achieve positive results I had to try different approaches including different plasmids with erythromycin resistance (pMSP-IRFP or pIAV7) and adjust protocols of tests.

The experiment with pMSP-IRFP had to be stopped due to non-reliable results. The fluorescence intensity obtained with Tecan Infinite® M1000 Pro Microplate Reader was too low to compare the data. Our suggestion is not to use two plasmids with nisin expression system inside one cell of *Lactococcus lactis* because the expression of genes from one or both of them may be inhibited.

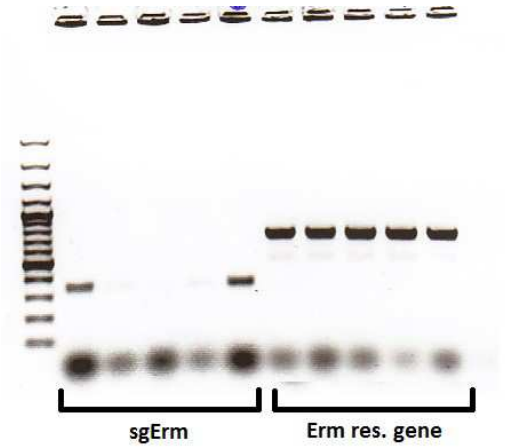


Figure 19. **Gel electrophoresis of the *L. lactis* colonies' DNA after sgErm gene and Erm resistance gene have been amplified using Taq PCR.**

Also there is a possibility that the cell is unable to maintain two relatively big plasmids inside its cytoplasm. To test that I performed a PCR on colonies to make sure sgErm (pNZ plasmid) and erythromycin resistance genes (pMSP plasmid) are present inside them. As it is visible on the gel (Figure 19) not all the colonies contain sgErm (pNZ plasmid) as they are supposed to.

The best approach turned out to be the expression test of *L. lactis* containing pIAV7 with multiple subculturing. *L.lactis* first needs time to grow in the medium free of erythromycin and nisin, then after some time induction with nisin is applied and the bacteria are left to grow while CRISPR-Cas9 system is active inside them. These induced bacteria are inoculated to the silid medium with erythromycin using drop plate method. After two days of incubation at 30°C the CFU are counted. To determine the number of viable colonies in the culture media it was necessary to compare two agar compositions: GM17C10E10 and GM17. The final results are demonstrated in the Figure 18.

There is a significant decrease in amount of cells treated with active form of Cas9 that are able to survive in the growth medium with erythromycin. I was not able to achieve 100% CRISPR-Cas9 efficiency, but I proved that it can be effective inside *Lactococcus lactis*.

4.2. Bacterial chromosome modification

4.2.1. Principle and aims.

The experiment was initiated in order to test CRISPR-Cas9 system in its ability to help in development of transgenic *Lactococcus lactis*. Design of the experiment is inspired by successful application of CRISPR in *Clostridium cellulolyticum* (Xu T et al., 2015).

The target of genetic modification is a gene of periplasmic serine protease HtrA (Poquet et al., 2000) that is a housekeeping protease of *L.lactis*. Its active part is located on the outer surface of the cell membrane and plays the role in extracellular proteolysis. This is an undesired characteristic of *L.lactis* if we plan to design this microorganism to produce recombinant proteins for targeted drug delivery. The wild-type protease is able to degrade proteins after or during their export to the cells surface. The bacteria with inactive form of the protease were already developed and were shown to be able to survive without its activity. Isolation of *L.lactis* cultures with inactive form of HtrA protease will not only prove the efficacy of CRISPR-Cas9 system in bacterial genome modification but also provide the strain of bacteria that will be suitable for further development of recombinant probiotics.

The principle of chromosome modification is based on homologous recombination (Figure 20) with an inactive form of HtrA protease cloned on the pNZ plasmid (see 3.2.1.3.), the same plasmid where the CRISPR system is located. Role of CRISPR-Cas9 system is to cut the wild-type gene of this protease in case when homologous recombination does not happen and the chromosome remains unchanged. Since *L.lactis* does not have means to seal the break created by Cas9 nuclease or Cas9D10A nickase, the unmodified bacteria should die.

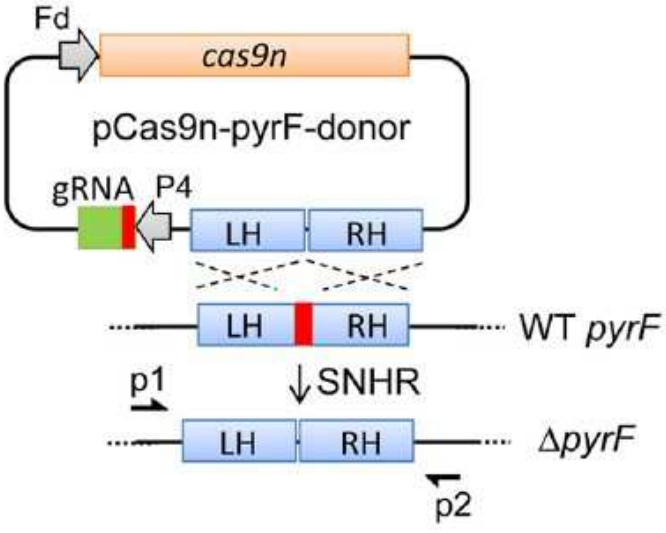


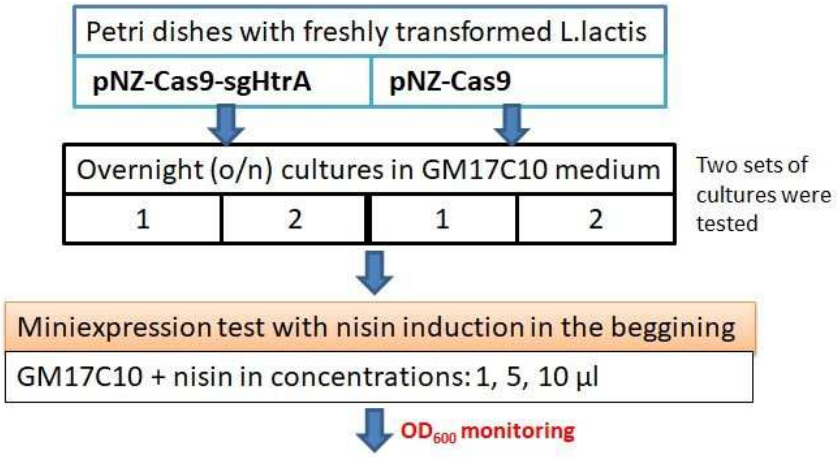
Figure 20. **Gene insertion into a chromosome based on the principle of homologous recombination. (**Xu T. et al, 2015)

Essentially the bacterium will be transformed with one plasmid (pNZ-Cas9D10A-sgHtrA-HtrAD) that serves two functions. The first is homologous recombination with the inactive protease gene which occurs naturally without induction. The second is Cas9 selective cleaving which only works after being induced with nisin in sub-inhibitory concentrations.

4.2.2. Primary test of Cas9 activity

Primary test was created to determine if the designed CRISPR system can kill the bacteria with a wild-type HtrA protease. *L.lactis* NZ9000 cells were transformed separately with two constructs pNZ-Cas9-sgHtrA and pNZ-Cas9 and grown in the liquid medium with nisin in the glass test tubes, while OD_{600} of the medium was regularly measured (Figure 21).

The plasmid I am testing in this experiment does not contain HtrAD fragment, therefore the cells cannot uptake the inactive form of HtrA protease and escape chromosome cleavage by Cas9 nuclease. Most of the bacteria containing induced pNZ-Cas9-sgHtrA should die and show lower OD_{600}.



After 3 hours of incubation 30°C						
	1st set of samples			2nd set of samples		
Nisin concentration µl	1	5	10	1	5	10
pNZ-Cas9-sgHtrA	0,180	0,172	0,164	0,208	0,196	0,176
pNZ-Cas9	0,296	0,284	0,272	0,396	0,312	0,304
After another 3 hours of incubation 30°C						
pNZ-Cas9-sgHtrA	0,272	0,272	0,268	0,280	0,252	0,268
pNZ-Cas9	1,352	1,960	1,108	1,616	1,332	1,192

Figure 21. **The protocol of the primary expression test of pNZ-Cas9-sgHtrA plasmid in *L.lactis*.**

In theory sgHtrA in a complex with Cas9 protein finds the HtrA protease gene in the bacterial chromosome and creates a double strand break which is lethal to a cell. That results in inhibited growth and lower OD_{600}. In the Figure 21 we can see that OD_{600} of the medium with *L.lactis* containing pNZ-Cas9-sgHtrA is on average 4.69 times lower than the control samples with pNZ-Cas9. This is a relevant proof that CRISPR array is functional to proceed to the next tests.

4.2.3. pNZ-Cas9D10A-sgHtrA-HtrAD miniexpression test in Tecan®.

In this test I wanted to obtain statistically relevant evidence of homologous recombination happening inside *L.lactis* and the influence of CRISPR-Cas9 system on selection of transgenic cells.

The test was performed in Tecan Sunrise® absorbance microplate reader (Figure 22). Four sets of samples (quadruples) with the different medium composition (with or without nisin) were loaded into the microplate. Samples incubation temperature was 30°C. The measurements of OD_{600} were performed every 20 seconds within one day.

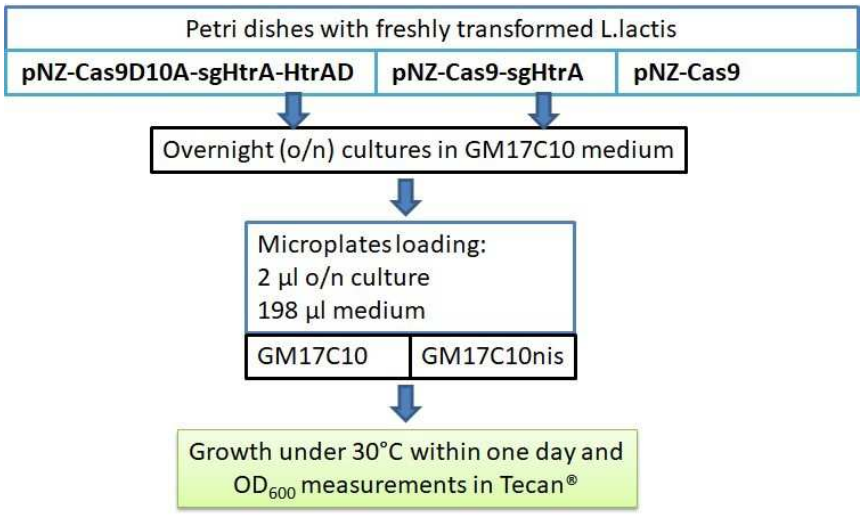


Figure 22. **The protocol of nisin controlled miniexpression test of pNZ-Cas9-sgHtrA-HtrAD in Tecan®.**

After the data had been collected I built and compared the growth curves (Figures 23, 24, 25) of samples with different pNZ plasmid constructs and their behavior with or without induction.

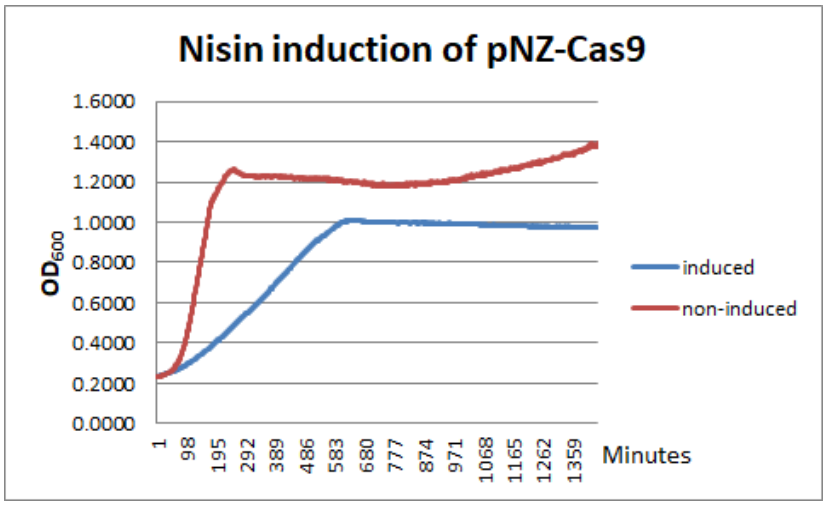


Figure 23. **Growth curves of *L. lactis* with pNZ-Cas9 in the presence or absence of nisin in the growth medium.**

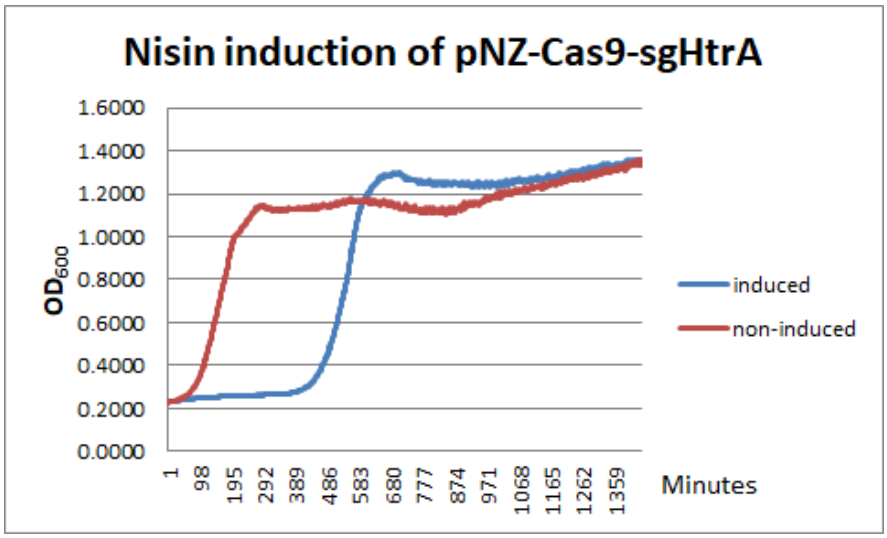


Figure 24. **Growth curves of *L. lactis* with pNZ-Cas9-sgHtrA in the presence or absence of nisin in the growth medium.**

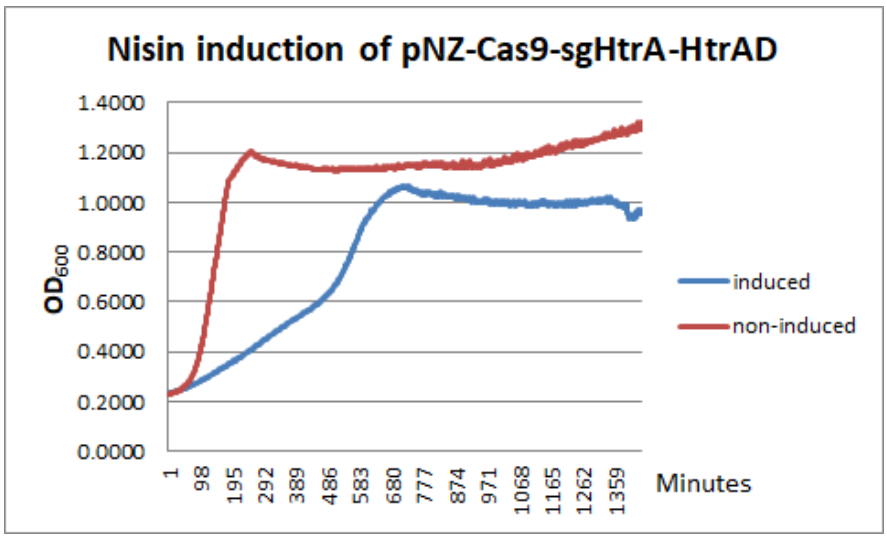


Figure 25. **Growth curves of *L. lactis* with pNZ-Cas9D10A-sgHtrA-HtrAD in the presence or absence of nisin in the growth medium.**

All non-induced constructs resulted in the same growth pattern (Figures 23, 24, 25) which means that the system was not working unless being induced.

Every plasmid construct demonstrates a unique behavior.

In Figure 23 induction of the samples with pNZ-Cas9 only resulted in a slightly inhibited growth rate due to nisin property to suppress bacterial growth.

On the figure 24 the induction of pNZ-Cas9-sgHtrA completely stops the bacterial growth for about 8 hours, which confirms the correct CRISPR function. The principle of this plasmid activity is described in the previous test (see 4.2.2.). But after 8 hours the surviving cells enable

the culture to grow exponentially again. It is likely that either some bacteria mutate and gain resistance to the action of Cas9 protein or they manage to lose the plasmid in some way.

And on the last graph (Figure 25) we can see a distinct biphasic growth pattern of the *L. lactis* samples with induced pNZ-Cas9D10A-sgHtrA-HtrAD. The first growth phase is slower due to some cells are being eliminated by Cas9 nickase while others perform homologous recombination. The second growth phase is faster because the culture reaches a critical amount of recombined bacteria that are able to reproduce without the risk of Cas9 induced breaks.

These results confirmed the activity of pNZ-Cas9D10A-sgHtrA-HtrAD and it is necessary to develop an efficient protocol for isolation of *L.lactis* with an inactivated protease in its chromosome.

4.2.4. Expression and modified strain isolation

In development of protocol for this experiment I met a lot of hurdles. After standard steps of transformed cell growth and induction I plated them on the agar plates containing nisin. The colonies that are expected to grow may have the desired modified genome. The main problem was to find this transgenic strain, using colony PCR and gel electrophoresis. In case there was any positive band on the gel is would be used for DNA sequencing. Eventually I was unable to identify a bacterium with a deletion in the protease gene in chromosome within limited amount of time. On the Figure 26 there is an example of typical protocol that I was trying to test.

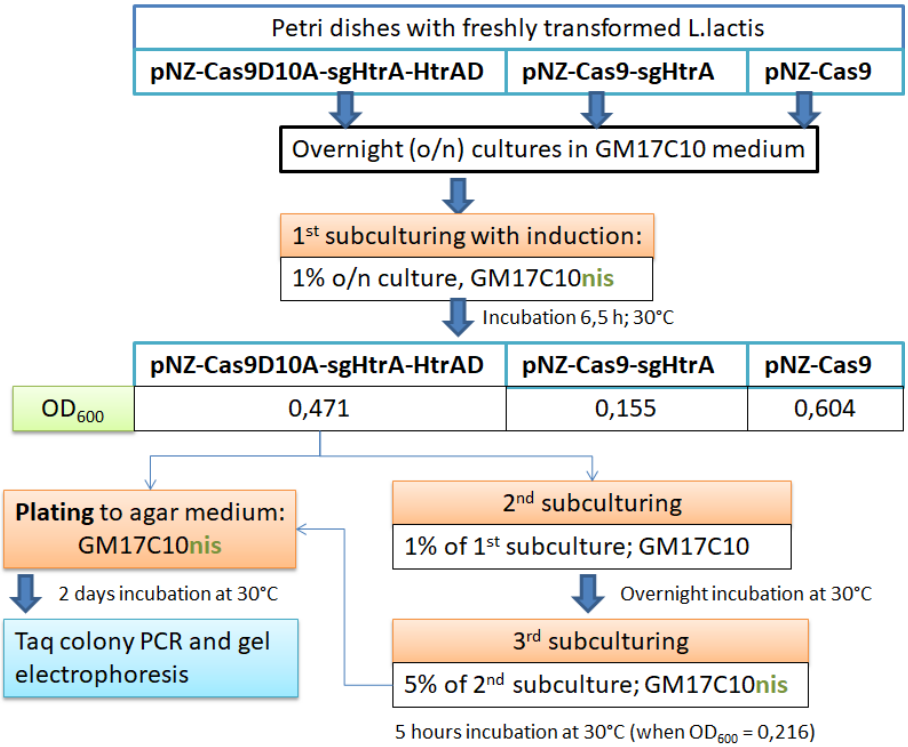


Figure 26. **Protocol for pNZ-Cas9D10A-sgHtrA-HtrAD plasmid nisin induction and positive strain isolation.**

When I got some colonies on the nisin agar plates it was necessary to test them with Taq colony PCR in Mastercycler® nexus X2 Eppendorf®. The primers that were used to test samples are

uHtrA-F, Htr-AB-r and Htr-del-R, that anneal in a way that the positive samples (with HtrA deletion from the protease gene) will only show one band on the gel and unmodified chromosome will give us two bands (Figure 27). The results of the protocol described on figure 26 are demonstrated on the picture of gel electrophoresis (Figure 28). The red arrow indicates a single positive colony which was used for the next test (KOD PCR).

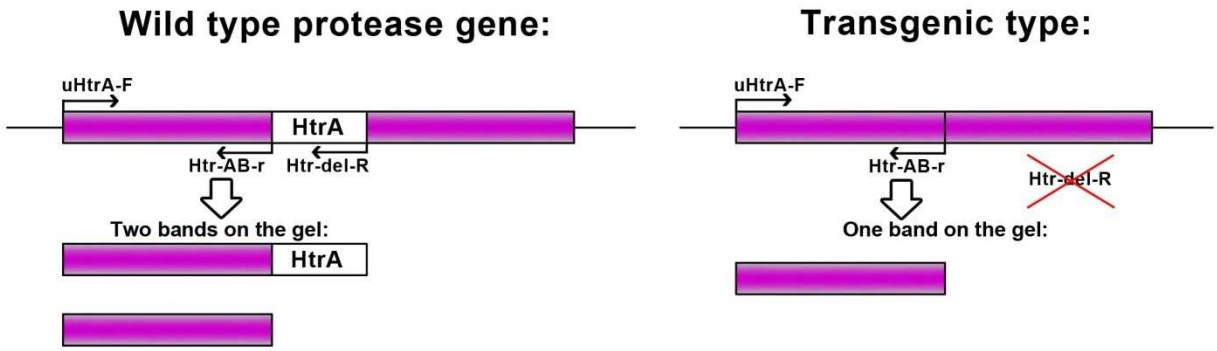


Figure 27. **Colony PCR and gel electrophoresis principle in the *L.lactis* chromosome**

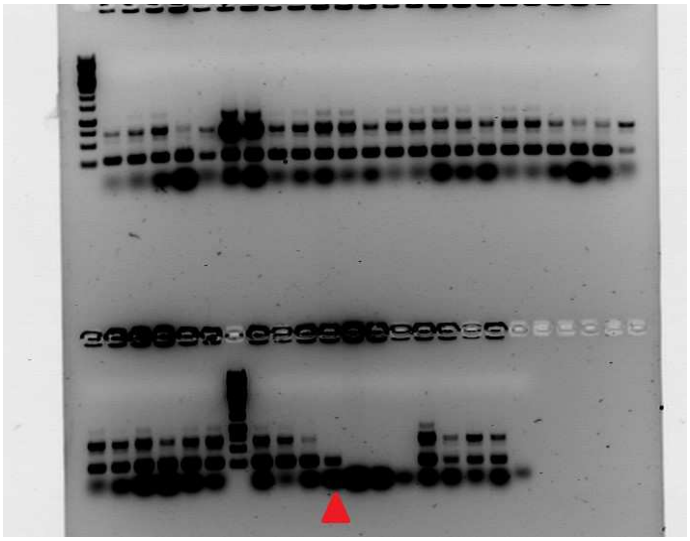


Figure 28. **Bacterial chromosome Taq PCR test results on agar gel**. 1 kb DNA ladder.

After I determined one colony with a single band I tested it further with proofread KOD PCR (Figure 29). Here I only used two primers: uHtrA-F and Htr-D-Sal that will amplify a shorter fragment (~1050 bp) in case the deletion happened in the chromosome. And if the chromosome remained unchanged the length of amplified fragment will be about 1300 bp. The sample showed a longer band on the gel, which means the HtrA fragment is still present in the protease and previous Taq PCR showed a false positive result.

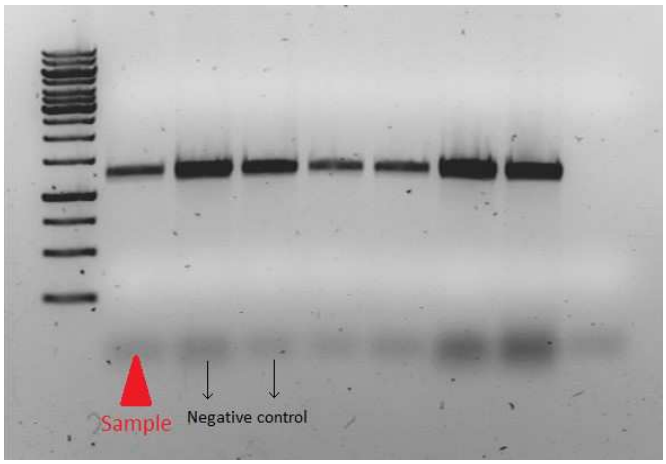


Figure 29. **Bacterial chromosome KOD PCR test results demonstrated on gel electrophoresis.** 1 kb DNA ladder.

In attempt to find modified colony of *L.lactis*, I tried to perform plating to nisin agar at the different steps of procedure. Freshly transformed cells with any of pNZ constructs do not grow on nisin plates, even if the nisin content in agar is reduced. Growth on these plates occurs only after bacteria have been cultured (and preferably induced) in the liquid medium before plating, which is reasonable due to growth suppressing properties of nisin.

4.2.5. Evaluation of results

As was demonstrated in the section 3.2.3. on the different growth curves, CRISPR-Cas9 system affects the growth behavior of *Lactococcus lactis* (Figure 30). I was not able to prove its precision and efficacy in this experiment because the transgenic strain was not found. More research is needed to optimize the expression protocol for transgenic strain isolation.

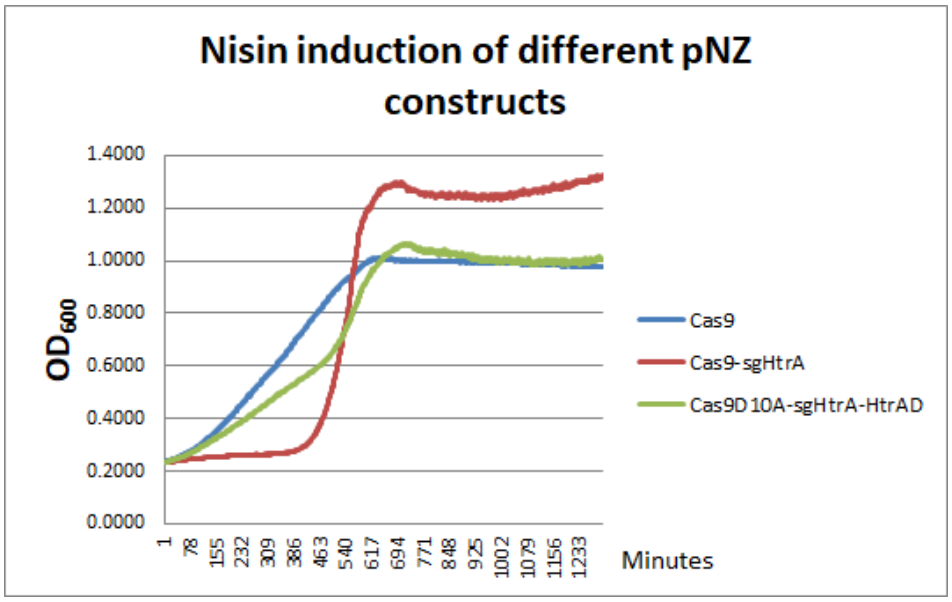


Figure 30. **Growth curves of *L. lactis* transformed with different constructs.** OD600 changes over the time (min.).

5. Discussion

Lactococcus lactis is a model microorganism used for research in different areas, but it is lacking an efficient tool for modification of its genome. This bacterium has a good potential for becoming a local delivery system for recombinant proteins in intestine (Berlec et al., 2012). Classical biotechnological methods, like homologous recombination, are available for prokaryotes, but are not perfectly suitable for this lactic acid bacterium. The main challenge is to isolate precisely a modified strain of *L.lactis* after homologous recombination has occurred.

After the emergence of CRISPR-Cas9 technology nobody has tried to test and develop it on *L.lactis* yet. So we decided to create a single-plasmid tool that will not only contain a homologous region but also a CRISPR system that will allow selecting a positive strain.

pNZ8148 was our vector of choice because it is a part of NICE system that has been effectively used in *L. lactis* for more than 20 years (Mierau I. et al, 2005). Earlier in our project, my colleagues have proven the successful application of dual protein expression in NICE system (Berlec et al., 2017), meaning that another nisin promoter PnisA was inserted in the MCS of pNZ plasmid, creating two nisin promoters. This enabled simultaneous transcription of sgRNA (instead of complex of crRNA and tacrRNA) and Cas9 gene.

Inspired by the research on Clostridium difficile (Xu et al., 2015) we decided to test both nuclease (Cas9) and nickase (Cas9D10A) activity and select the most suitable protein for *L.lactis*, but the comparison was not possible due to problems with cloning. Further research on Cas9D10A is required. In my work Cas9 has been proven to be functional.

During my experiments I constructed and cloned the functional ready-to-use pNZ plasmid for genetic engineering of *L. lactis*. Besides original components it also contained targeted sgRNA, second nisin promoter, Cas9 gene and homologous region. The CRISPR array showed its activity inside *L.lactis* in the plasmid curing test (see 4.1.). But despite the evidence on its activity, it was still impossible for me to isolate the desired transgenic strain of *L.lactis*. In my opinion the protocol of cell induction and cultivation should be further optimized to yield higher percent of positive colonies. Optimizations might be done in numbers of subcultures, time of cultivation, order of induction, antibiotic content of the different growth medium.

The full description of the project I have been working on is submitted for publication under the title "Single plasmid systems for inducible dual protein expression and CRISPR/Cas9 gene regulation in lactic acid bacterium Lactococcus lactis." (Berlec et al., 2017).

6. Materials and methods

6.1. Methods

Colony Taq PCR: 10X DreamTaq buffer, 0.5% DreamTaq DNA polymerase and 16% dNTP from ThermoFisher® scientific.

1 µl of each primer of concentration 5 µM; distilled water – rest of the volume; sample colony at the end of the tip.

Note: the polymerase is added after the cell lysis step (99°C – 10′).

PCR protocol:

99°C – 10′
50°C – 2′
94°C – 30′′
46°C – 1′
72°C – 1′ } x30
72°C – 5′
4°C – ∞

Taq PCR: 10X DreamTaq buffer, 0.5% DreamTaq DNA polymerase and 16% dNTP from ThermoFisher® scientific.

1 µl of each primer of concentration 5 µM; distilled water – rest of the volume; DNA sample 0.25-1 µl (depending on concentration).

PCR protocol:

94°C – 5′
94°C – 30′′
48°C – 1′
72°C – 1′ } x30
72°C – 5′
4°C – ∞

KOD colony PCR: 10X KOD buffer, 2% KOD Hot Start DNA polymerase and 10% dNTP from Merck Millipore®.

~6% of each primer of concentration 5 µM; distilled water – rest of the volume; sample colony.

Note: the polymerase is added after the cell lysis step (99°C – 10′).

PCR protocol:

99°C – 10′
50°C – 2′
95°C – 20′′
48°C – 10′′
70°C – 30′′ } x30
70°C – 2′
4°C – ∞

DNA isolation and purification:

For DNA isolation and purification I used two kits – NucleoSpin® Plasmid and NucleoSpin® Gel and PCR Clean-up by MACHEREY-NAGEL.

Restriction digestion:

For 50 µl reaction mixture – 2000 ng of the fragment to restrict, 2 µl of each restriction enzyme, 5 µl of 10X FastDigest buffer ThermoFisher®, rest of volume – distilled water. Procedure:

restriction reaction mixture is incubated at 37ºC for 2 hours, afterwards the sample is loaded to the gel together with a loading dye.

Gel electrophoresis:

Gel composition for 50 ml: 1% agarose solution in TAE buffer; 5 µl SYBR® Safe DNA Gel Stain.

DNA ladder: 1 µl of 100 bp or 1kb of GeneRuler DNA Ladders, 2 µl 10X Loading Dye by ThermoFisher®, 9 µl distilled water.

Power sourse set to : 90 – 120 V.

Ligation reaction:

For 50 µl total reaction mixture: 5 µl T4 DNA Ligation buffer, 1,5 µl T4 DNA Ligase by ThermoFisher® , amounts of purified vector and insert (or fragments) were calculated using on-line ligation calculator (The University of Texas at Dallas, 2010).

6.2. Reagents

KOD DNA Polymerase Merck Millipore

M17 broth acc. to TERZAGHI for microbiology EMD Millipore corporation

NucleoSpin Gel, NucleoSpin Plasmid and PCR Clean-up kits from Macherey and Nagel

Phusion Hot Start II DNA Polymerase from Thermo Fisher

Restriction enzymes (Fast Digest) from Thermo Fisher

T4 DNA ligase from New England Biolabs

Taq DNA Polymerase from Thermo Fisher

6.3. Equipment and devices

Biorad Genepulser® II

Eppendorf Centrifuge 5424

Eppendorf Research® automatic pipettes

LAMBDA Bio+ Spectrophotometer (PerkinElmer®)

Mastercycler® nexus X2 Eppendorf®

NanoDrop™ 2000c Spectrophotometer (Thermo Scientific™)

Tecan Infinite® M1000 Pro Microplate Reader

Tecan Sunrise® absorbance microplate reader

7. References:

Berlec, A., Ravnikar, M., Strukelj, B. (2012). Lactic acid bacteria as oral delivery systems for biomolecules. Die Pharmazie, 67(11):891-898.

Berlec, A., Škrlec, K., Kocjan, J., Olenic, M., Štrukelj, B. (2017). Single plasmid systems for inducible dual protein expression and CRISPR/Cas9 gene regulation in lactic acid bacterium Lactococcus lactis. Submitted for publication.

Braat, H., Rottiers, P., W.Hommes, D., Huyghebaert, N., Remaut, E., Remon, J., J.H. van Deventer, S., Neirynck, S., P.Peppelenbosch, M., Steidler, L. (2006). A Phase I Trial With Transgenic Bacteria Expressing Interleukin-10 in Crohn's Disease. Clinical Gastroenterology and Hepatology, 4: 754 – 759.

Bryan, E.M., Bae, T., Kleerebezem, M., Dunny, G.M. (2000). Improved vectors for nisin-controlled expression in gram-positive bacteria. Plasmid: 44(2):183-90.

Choi, K.R., Lee, S.L. (2016). CRISPR technologies for bacterial systems: Current achievements and future directions. Biotechnology Advances, 1180-1209.

Chylinski, K., Makarova, K.S., Charpentier, E., Koonin, E.V. (2014). Classification and evolution of type II CRISPR-Cas systems. Nucleic Acid Research, 42: 6091 – 6105.

Guandalini, S. (2011). Probiotics for Prevention and Treatment of Diarrhea, Journal of Clinical Gastroenterology, 45: S149-53.

Jacob Corn https://innovativegenomics.org/blog/how-to-make-a-guide-rna-for-cas9/

Jinek, M., Chylinski, K., Fonfara, I., Hauer, M., Doudna, J.A., Charpentier, E. (2012). A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science, 337: 816-821.

Laroute, V., Tormo, H., Couderc, C., Mercier-Bonin, M., Le Bourgeois, P., Cocaign-Bousquet, M., Daveran-Mingot, M.L. (2017). From Genome to Phenotype: An Integrative Approach to Evaluate the Biodiversity of Lactococcus lactis. Microorganisms, 5(2): 27.

Mierau, I., Kleerebezem, M. (2005). 10 years of the nisin-controlled gene expression system (NICE) in Lactococcus lactis. Applied microbiology and biotechnology, 68(6):705-717.

Mo Bi Tech. (2008). NICE® Expression System for Lactococcus lactis.

Natural competence - https://en.wikipedia.org/wiki/Natural_competence

Perez-Arellano, I., Zuniga, M., Perez-Martinez, G. (2001). Construction of Compatible Wide-Host-Range Shuttle Vectors for Lactic Acid Bacteria and Esherichia coli. Plasmid, 46: 106-116.

Poquet, I., Saint, V., Seznec, E., Simoes, N., Bolotin, A. and Gruss, A. (2000). HtrA is the unique surface housekeeping protease in *Lactococcus lactis* and is required for natural protein processing. Molecular Microbiology, 35: 1042–1051.

RestrictionMapper – on-line restriction site analysis http://www.restrictionmapper.org/

Steidler, L., Hans, W., Schotte, L., Neirynck, S., Obermeier, F., Falk, W., Fiers, W., Remaut, E. (2000). Treatment of Murine Colitis by Lactococcus lactis Secreting Interleukin-10. Science, 289: 1352-1355.

The University of Texas at Dallas. (2010). Ligation Calculator.

Xu, T., Li, Y., Shi, Z. (2015). Efficient Genome Editing in Clostridium cellulolyticum via CRISPR-Cas9 Nickase. Applied and Environmental Microbiology, 81(13):4423-4431.