Lactic acid bacteria as producers of pharmaceutical-grade molecules: genetic engineering tools for improvement of *Lactococcus lactis* strains

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I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

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Resumo

As bactérias ácido lácticas (LAB) são uma ferramenta promissora para a produção de DNA e proteínas de grau farmacêutico ou como vetores para libertação destas moléculas *in vivo*. A necessidade de estirpes otimizadas para estas aplicações tornam essencial descobrir estratégias para edição genómica em LAB. Duas abordagens baseadas em *recombineering* (acoplado ou não a um sistema CRISPR-Cas9) foram otimizadas e aplicadas para inativar o gene *nth* (endonuclease) no genoma de *Lactococcus lactis* LMG19460. Na estratégia Reisch & Prather, foram clonados a origem de replicação pAMβ1 e o gene de resistência a eritromicina no plasmídeo com o gene *Cas9* e o sgRNA para *nth* foi introduzido no plasmídeo que contém os genes *λ-Red*. No entanto, o primeiro plasmídeo (pCas9cr4) sofreu alterações após transformação em *L. lactis* e, assim, o segundo (pKDsgRNA-nth) não foi introduzido nas células. Na estratégia de Datsenko & Wanner, o plasmídeo com os genes *λ-Red* (pKD46) foi introduzido na estirpe com sucesso. A integração da cassete de resistência a canamicina na região do gene *nth*, não foi alcançada. A dificuldade desta estirpe em crescer com L-arabinose como única fonte de carbono, torna difícil a indução de expressão das proteínas λ-Red. Adicionalmente, *L. lactis* LMG19460 tem elevada resistência a canamicina, tornando a seleção de transformantes complicada. Apesar dos passos de otimização alcançados, dado que a estirpe tem uma elevada taxa de degradação de DNA exógeno, é provável que os plasmídeos e DNA linear não se mantenham estáveis na célula tempo suficiente para que a próxima molécula de DNA da estratégia seja introduzida.

Palavras-chave: Bactérias ácido láticas, DNA plasmídico, recombineering, CRISPR-Cas9, engenharia de estirpes.

Abstract

Lactic acid bacteria (LAB) are a promising tool in new biomedical approaches as producers of pharmaceutical-grade DNA and proteins and live vectors for delivery of these molecules. The need for optimized strains for these applications makes it essential to find appropriate genome editing tools for LAB. In this work, optimization and application of two recombineering-based approaches (coupled or not to a CRISPR-Cas9 system) were done with intent of inactivating the endonuclease *nth* gene from *L. lactis* LMG19460. For the Reisch & Prather strategy, the pAMβ1 ori and the erythromycin resistance gene were cloned in a plasmid carrying the *Cas9* gene and the sgRNA targeting *nth* was cloned into a plasmid carrying λ-Red proteins. However, the first plasmid (pCas9cr4) suffered alterations upon transformation into *L. lactis* and therefore the second (pKDsgrRNA-nth) was not introduced in the cells. To implement the Datsenko & Wanner strategy, the plasmid carrying the λ-Red recombineering genes (pKD46) was successfully introduced. But integration into the genome of the kanamycin resistance cassette targeting the *nth* gene was not achieved. This could be due to the difficulty of the strain to use L-arabinose as the only carbon source, making it troublesome to induce expression of the λ-Red proteins. In addition, *L. lactis* LMG19460 has high resistance to kanamycin, making selection of recombinants arduous. Although some optimization steps were achieved, since the strain has a high rate of exogenous DNA degradation, it is likely that the plasmids and linear DNA do not stay intact in the cells enough time to introduce the next piece of DNA in the strategy.

Key-words: Lactic acid bacteria, plasmid DNA, recombineering, CRISPR-Cas9, strain engineering.

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List of Abbreviations

bp	base pair
Cas	CRISPR associated protein
CPEC	Circular Polymerase Extension Cloning
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR-RNA
DNA	deoxyribonucleic acid
DSB	Double-strand breaks
dsDNA	double-strand DNA
eryR	erythromycin resistance
FDA	Food and Drug Administration
FLP	Flippase
FRT	FLP recognition target
gDNA	genomic DNA
GIT	Gastrointestinal tract
GM-17	M-17 broth supplemented with 0.5% glucose
GMO	Genetically Modified Organism
GRAS	Generally Recognized as Safe
HDR	Homology-directed repair
HIV	Human Immunodeficiency Virus
HR	Homologous Recombination
LAB	Lactic Acid Bacteria
LB broth	Luria-Bertani broth
LPS	Lipopolysaccharides
MRS medium	De Man, Rogosa and Sharpe medium
NHEJ	Non-homologous end joining
no-SCAR	Scarless Cas9 Assisted Recombineering
nt	nucleotides
OD	Optical Density
PAM	Protospacer adjacent motif
PCR	Polymerase Chain Reaction
pDNA	plasmid DNA
RNA	Ribonucleic acid
sgRNA	Single guide RNA
SpCas9	*Streptococcus pyogenes* Cas9
ssDNA	Single-strand DNA

1. Introduction

1.1. Lactic acid bacteria

Lactic acid bacteria (LAB) constitute a heterogeneous group of Gram-positive bacteria that produce lactic acid as the major end product of sugar fermentation. They are acid tolerant, catalase negative, anaerobic aerotolerant bacteria and can either be cocci or rod-shaped. This group includes species from *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus* genera[1]. Most microorganisms included in this group are non-pathogenic and non-invasive. Lactic acid bacteria have restricted biosynthetic capabilities therefore requiring rich growth media and niches in which amino acids, peptides, fatty acids, vitamins, purines, pyrimidines and sugars are available[1,2]. Thus, they can be found in fermented foods and beverages, plants, sewage and in many mucosal compartments in human and animal bodies[1,3].

Most LAB are classified as Generally Recognized As Safe (GRAS) by the United States Food and Drug Administration (FDA) due to their historically safe use in food and beverage fermentation and their contribution to the healthy microflora of human mucosal surfaces[4]. The physiology and status of LAB has contributed to their extensive applications in several industries, especially in food fermentation. However, the characteristics and genetics of these bacteria turn them into potential tools for new pharmaceutical and therapeutic approaches. Their natural beneficial traits allied with the available genome editing tools makes it possible to create new strains for new applications and products: as cell-factories for plasmid DNA (pDNA) and heterologous protein production, live delivery vectors for therapeutics, as well as improved strains for their traditional application in food and as probiotics[5].

The model and best characterized LAB is *Lactococcus lactis,* a non-pathogenic, non-sporulating, non-motile species used in the production of fermented dairy products, with GRAS status. Their well-adapted homofermentative metabolism of lactose makes dairy products the best and most documented habitat for lactococci. This species has three sub-species: *L. lactis* spp. *lactis* and *L. lactis* spp. *cremoris* have received the most attention because they are used as important starter cultures in dairy industries, and *L. lactis* ssp. *hordniae* with a *L. lactis* spp. *cremoris* genotype and a *L. lactis* spp. *lactis* phenotype[6]. *L. lactis* ssp. *lactis* IL1403 was the first LAB strain that had its genome sequenced and it is commonly used in laboratory[7].

1.1.1. Current lactic acid bacteria applications

Several products can be fermented from LAB: dairy products, vegetables, cereal-based products and even meat, and different species tend to dominate specific fermentation environments or niches[2,3]. The major focus, however, has mostly been on species related with dairy industries that are used as starter cultures in dairy fermentations, to accelerate and direct the process. Their physiology and metabolism have several beneficial traits for the food industry. Firstly, production of lactic acid leads to acidification of the media which inhibits the growth of many pathogenic microorganisms, allowing preservation. Some LAB also improve shelf-life of the fermented products by secreting bacteriocins

which are peptides with antimicrobial activity against closely related bacterial strains (nisin A, for instance)[1,6]. Others can add taste (from enzymes, natural sweeteners or vitamins) or texture (from exopolysaccharides) to the fermented food[3,8]. In most cases, the starter cultures for dairy products fermentation contain *L. lactis* spp. *cremoris* and *L. lactis* spp. *lactis*. The need for understanding these strains made the *Lactococcus lactis* the best studied and characterized species of LAB. For the food industry, regulation prohibits the use of Genetically Modified Organisms (GMOs) so the best suited traits for the industry are naturally transferred and selected without genetic modification[5].

Nevertheless, LAB have also several medical applications. The most common, as probiotics, living microorganisms that confer a health benefit on the host (FAO/WHO, 2002). Due to their GRAS status and ability to traverse through the gastrointestinal tract (GIT) of mammals, LAB from the genera *Lactobacillus* and *Bifidobacterium*, have been used as probiotics to improve health[4]. Their ability to lower the pH and sometimes produce antimicrobial molecules makes them good regulators of GIT microflora[3]. Probiotics have been studied mostly against gastrointestinal disorders, but LAB have revealed to have several beneficial effects on health. They also have the potential to lower cholesterol levels, stimulate and improve immune response, and even have antitumor, antimutagenic and anticarcinogenic activity (although clinical evidence is still lacking)[3,4].

The physiology and status of LAB, the increasing number of strategies for engineering of microorganisms and the need for new approaches in certain fields has created new potential applications of LAB in industry and medicine. However, the new arising pharmaceutical applications need the use of modified strains for an efficient treatment, cure or prevention of diseases[2]. LAB are a promising tool for use in: 1) DNA production for DNA vaccines, 2) recombinant protein and metabolite production; 3) live mucosal vaccination (as DNA carriers or antigen producers).

1.1.2. Lactic acid bacteria as cell factories for production of plasmid DNA and recombinant proteins

Plasmid DNA can carry any gene of interest for food industries and even for medical purposes. Its production, however, must be held inside live microorganisms, being important to choose the best host according to the intended use of pDNA. Food- and pharmaceutical-grade pDNA has to be produced under strict regulations so that its administration is safe for humans. Therefore, it is imperative that the host has the necessary characteristics for efficient production of good quality pDNA, or that the host can be engineered into having such traits[9].

Currently, pDNA is mainly produced using *Escherichia coli* strains and plasmid backbones that are known to be effective in the production of recombinant proteins. However, these are not optimized for production of pharmaceutical-grade pDNA. Although the use of *E. coli* is optimized for higher levels of protein production, it lacks an efficient protein secretion system and, since they are Gram-negative bacteria, they produce lipopolysaccharides (LPS), toxic to humans, which leads to difficult and expensive downstream purification processes[9]. Gram-negative bacteria safe and high yielding production is hindered by their complex secretion system that adds the necessity of more intricate steps of purification[9,10].

Since Gram-positive do not produce LPS in their cell wall, there is no possible immunogenic response due to co-purification with the desired proteins[11], making them ideal for pharmaceutical-grade pDNA production. LAB such as *L. lactis*, are a sturdy alternative for pDNA production when it comes to other Gram-positive models, such as *Bacillus subtilis*. The main problem with the latter is that it has a complex extracellular proteolytic system that degrades many of the secreted heterologous proteins[11]. Although mutant *Bacillus* strains have been engineered to be defective for some of the extracellular proteases in this system, extracellular proteolysis hasn't been completely abolished, only decreased[11]. Whereas, in *L. lactis* laboratory strains there is only one exported housekeeping protease, HtrA, and a protease-free mutant is available[11]. The complex secretion system of *Bacillus* strains also makes the downstream purification process of protein production more difficult. *L. lactis*, however, only secrete to the medium, in measurable levels, one major protein, Usp45[11]. Thus, LAB cover several advantages as a cell factory system for pDNA and recombinant protein production.

For production of food- and pharmaceutical-grade pDNA and recombinant proteins it is imperative to work with plasmid-free bacteria, such as *L. lactis* ssp. *lactis* IL1403 and *L. lactis* ssp. *lactis* LMG19460 (which had its genome recently sequenced by Silva, *et al.*, 2017[12]). These strains originate from parental strains isolated from starter cultures of cheese factories. Most of the traits that make *L. lactis* strains advantageous for dairy products industries are plasmid encoded[13]. However, the presence of some of these traits is disadvantageous when It comes to heterologous plasmid and protein production because it allows for degradation of exogenous plasmids and proteins. Furthermore, the presence of endogenous plasmids can cause conflict with the exogenous/desired plasmids we wish to produce if the two plasmids are incompatible, i.e., the plasmids fail to be stably co-inherited, meaning one of them will be lost. A higher yield of production can also be achieved since metabolic efforts are put only in the replication of the plasmid of interest and production of the desired recombinant protein, instead of being shared with endogenous plasmids and the products of their encoded genes. Production and secretion systems of LAB have been studied and engineered, leading to the successful production of reporter molecules, antigens (viral, bacterial and eukaryotic), interleukins, viral proteins, bacteriocins and enzymes[10,11,14].

Research findings regarding genetic factors linked to several diseases, allied with the discovery of recombinant DNA technology and gene cloning, lead to the creation of new biomolecular and biotechnological approaches to certain diseases. Gene therapy and DNA vaccines are therapeutic approaches in which nucleic acids are transferred to the somatic cells of the patient with resulting therapeutic effects[15,16]. This principle offers new therapeutic possibilities for diseases without or with less effective treatments: monogenic disorders (such as cystic fibrosis), and also more complex diseases (such as cancer, autoimmune diseases, diseases of the nervous system or cardiovascular diseases)[16,17]. Gene therapy poses several advantages over other medical strategies for acquired and inherited diseases, such as: correction of the genetic cause of the disease, selective treatment of target cells or tissues and long-term treatment[15,17]. Strict regulations and safety concerns lead to several findings and strategies around this technology, aimed at developing effective and safe methods of delivering and expressing heterologous genes in target cells.

Traditional vaccines are composed of dead or attenuated pathogens or their subunits. Non-live vaccines usually do not provide long-term immunity, whereas live-attenuated vaccines raise some safety concerns such as reversion of the pathogen's virulence. DNA vaccines enable us to deliver specific genes to target cells, where production of the desired protein takes place. These genes can encode antigens that are then produced in the cells stimulating potent cellular and humoral immune responses[18].

The therapeutic DNA molecule can be delivered by an *in vivo* or *ex vivo* approach. The first, consists in the direct injection of the DNA into the patient, while the *ex vivo* approach relies on *in vitro* modification of autologous cells of the patient that are re-introduced into the patient's body. Delivery of DNA therapies must be thoroughly controlled so that the pDNA of interest acts in the target site and is produced in enough concentration to show effective therapeutic result. For successful gene therapy, the desired gene must be recognized and transported inside the nucleus of the target cell without being degraded or generate an immune attack[16,17]. Injection of the naked DNA molecule has low efficiency as a clinical approach, thus, the gene encoding the desired protein should be delivered by different molecules or methods. The most studied methods use vectors, viral (retroviral, adenoviral and adeno-associated) or non-viral (naked plasmids or inside polymer carriers), for gene delivery, as they can be produced and purified easily and in high concentrations in bacterial hosts[16]. Viral vectors are the most used since they are more efficient in introducing and prompt expression of the DNA in the nucleus of the target cell. But, these vectors bring several safety concerns due to potential recombination and integration of the gene in the genome of the host cells which can have oncogenic effects, and possible unexpected immune responses[16,17]. Non-viral vectors are safer alternatives to viral vectors but their naked injection in the host lead to relatively low levels of expression. There are several strategies to improve delivery of therapeutic genes: increase DNA stability (reducing plasmid size, for example), decrease safety concerns (the use of minicircles allows the delivery of plasmids with reduced prokaryotic elements) and optimize plasmid transfection (chemical carriers can deliver the DNA protecting it from degradation and potential host immune attack)[16,17]. All these disadvantages, however, can be avoided using alternatives that can carry bigger sized DNA molecules, do not have pathogenic effects and can deliver the therapeutic molecules undegraded into the target cells. This can all be achieved by using live bacteria engineered to safely carry the therapeutic gene and express it in the target site[16].

1.1.3. Lactic acid bacteria as delivery vehicles of therapeutic molecules

Attenuated pathogenic bacterial strains of *Shigella*, *Listeria*, and *Salmonella* have already been used for delivery of therapeutic molecules into mammalian cells but are associated with safety concerns due to the possible reversion to pathogenicity[19]. Due to their GRAS status, and non-invasive and non-colonizing nature, LAB are a safer alternative. Studies have been done on the use of modified probiotic LAB strains for delivery of tumor suppressor molecules to cancerous tissue based on their ability to translocate from the GIT to the blood supply and from there to the tumor[20]. The hypoxic environment and resistance to immune attacks within the tumor, allows the growth of anaerobic bacteria, such as LAB. Engineered strains could deliver drugs to directly extinguish the tumor or reverse their evasiveness to the hosts immune system.

Delivery of therapeutics by live bacteria can be used as a strategy for vaccination to prevent infectious diseases and treat autoimmune disorders and cancer[19]. The administration of therapeutic molecules through mucosal routes allows mucosal but also systemic immunity, with enhanced potency and specificity and less side effects[21,22]. Mucosal vaccination poses several advantages over systemic vaccines against infections or diseases of mucosal origin. Their enhanced potency and specificity allied with lesser side effects, makes them a safer alternative to systemic and attenuated vaccines. Furthermore, it is administrated in a non-invasive way and without need of trained personnel (important trait for mass vaccination programs)[21]. The use of live engineered bacteria for mucosal delivery of therapeutic genes allows efficient and safe immunization.

Live engineered LAB can deliver and produce directly into the appropriate site, antigen genes encoded in pDNA. Lactic acid bacteria, such as *L. lactis*, have been widely studied for delivery of DNA therapies, production and presentation of antigens at target sites. Their GRAS status and ability to traverse through the GIT and other mucosa of animals and humans without host immune response, associated with the available strain engineering methods, makes them a safe and effective option for use in mucosal vaccination[19,22]. Several studies have reported engineered *L. lactis* strains as potential live mucosal vaccines with ability to produce a different number of proteins: antigens derived from bacteria, viruses, and parasites, as well as cytokines[21]. The most prominent being the treatment, in phase I of clinical trials, of Crohn's disease, by mucosal vaccination with a modified *L. lactis* strain that produces *in situ* human interleukin-10 (IL-10), reducing inflammation[21,23]. Other study shows the potential of engineered lactococcal strains to deliver Bovine β-lactoglobulin (allergen present in milk) antigen to mammalian epithelial cells[19]. More recently, *L. lactis* has been tested as an orally administered vaccine platform for immunization against the Human Immunodeficiency Virus (HIV). The HIV-1 antigen is anchored to the cell wall and has resulted in potent humoral and cellular immune responses in mucosal and systemic compartments of mice[24]. Although several studies indicate LAB as potential live delivery vectors of antigens, some mechanisms need to be improved, such as: antigen stabilization by down regulation of housekeeping protease genes, better protein secretion by overexpression of chaperones or improvement of anchoring the protein to the cell wall, and overall improvement in the interactions between the colonizing LAB and the host immune system[25]. The engineering and optimization of pDNA production and delivery by LAB has been assessed, turning the use of LAB as live delivery vectors of therapeutics, a promising strategy, but with still room for improvement.

1.2. Improving lactic acid bacteria for heterologous pDNA and protein production

The range of potential uses of LAB is impressive, however, several improvements are needed to obtain optimized strains for the desired applications. Growth and media conditions can be optimized up to a certain degree, but these need to be coupled to more profitable strategies. Moreover, when the target is *in situ* production of therapeutic molecules it is necessary to have the best suited strains to obtain clinically effective concentrations.

Numerous studies have shown that there are already available tools for higher yielding protein production and delivery by LAB. Several constitutive and inducible gene expression systems have been developed in LAB and delivery systems targeting different cellular locations allow easy secretion and recovery of proteins[14,21]. For improved pDNA production, strategic modifications of promoters or plasmid replicons have been attempted and wide host-range high plasmid copy number replicons are available[26]. There are still, however, some setbacks for optimized food- and pharmaceutical-grade pDNA and protein production. Since most GRAS status LAB strains, which are used in the food industry, are wild-type (the use of genetically modified organisms is frowned upon), there are several disadvantageous traits that need to be modified for heterologous protein production. The silencing of non-essential genes coding these traits, or the overexpression of advantageous genes is then necessary, for a profitable use of LAB as cell-factories for pDNA and recombinant protein production.

As cell factories for protein production it is essential that LAB strains are optimized to yield high concentrations of the desired product. In most studies reporting heterologous protein production by LAB, the protein is found only in one cellular location (intracellular, extracellular or cell wall-anchored). Comparing the production yields in each one, it was concluded that secretion lead to the highest levels of protein concentration. This suggest that heterologous proteins tend to suffer proteolysis in the cytoplasm, while by secretion the proteins can evade degradation[10]. Although protease-free laboratory strains are available for *L. lactis*[11] and heterologous proteins can be fused with specific homologous signal peptides that target and increase secretion into the media[10], secretion of the desired protein is not an universal solution. Firstly, there are still intracellular enzymes with proteolytic functions (peptidases, housekeeping proteases) present in *L. lactis* that could impair protein production[10]. Secondly, the different potential applications of LAB, especially in the medical approaches, are not always benefited by secretion of proteins[27]. For use of live bacteria for vaccination, the display of foreign antigens on the surface of cells allows a direct contact of between the antigen and the host's immune system[28]. Finally, a major bottleneck in the use of LAB as cell factories is related to pDNA production.

For high yield protein or even pDNA production, it is necessary to maximize pDNA concentration. These setbacks can be surpassed by modification of bacterial strains to optimize biomass and plasmid production or decrease of plasmid degradation. This can be achieved with genome editing tools by eliminating non-essential genes or overexpressing others related to these traits. Previous studies and literature report relevant genes that when knocked-out could improve pDNA quantity and quality, these can include: endonucleases (such as the endonuclease encoded by the *nth* gene)[7], recombinases (*recA*)[29] and proteases (*htrA, clpP*)[30,31] that degrade the desired products, genes that are linked to biomass production, sugar metabolism or to nucleotide production (*pgi, pyk*)[32,33].

For this type of strain optimization, there are several tools available and experimental work going on in LAB.

1.2.1. Genetic engineering in lactic acid bacteria

Lactic acid bacteria are a promising tool for several different industries, but the need for their optimization is still existent. Natural methods of DNA delivery are chosen to avoid the use of GMOs in

the food industries, however, for creating strains for therapeutic use it is not possible to run from genetically modified LAB. The engineered strains can currently be modified by means of homologous recombination (HR) or recombineering, and these can be coupled to technologies that allow easy counter-selection of the mutant cells without the use of markers, such as CRISPR-technology and Cre-*lox* or FLP-FRT systems[5].

1.2.1.1. Recombineering strategies

Recombineering, as opposed to plasmid-based HR, eliminates the need for double crossovers. It is a strategy developed in *E. coli* for recombination-mediated genetic engineering that allows insertion, deletion or alteration of sequences but does not depend on location of restriction sites. Linear DNA, double- (dsDNA) or single-stranded (ssDNA) is introduced by electroporation in the cells. Initial studies focused on the use of this DNA as substrate for three proteins derived from the lambda-red (λ-Red) phage: Beta, Gam and Exo (Figure 1)[34].

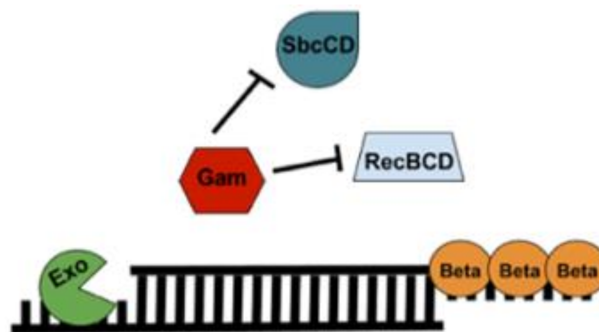


Figure 1. Components of the λ-Red phage recombineering system: Gam prevents hosts nucleases from digesting the linear DNA (in *E. coli* – SbcCD and RecBCD nucleases), Exo degrades dsDNA from 5' to 3', Beta protects the ssDNA created by Exo and promotes annealing to the target sequence in the cell. Figure from Kenkel (2016)[35]

These proteins have to be expressed and functional in the cell for recombination to occur, which can be a difficulty since these components are strain specific, that is, suitable versions of these proteins have to be available; other system for example, uses RecT system from the Rac prophage[5,34]. When functional, Beta is a ssDNA-binding protein (recombinase), Gam supresses hosts nucleases and Exo is a 5'–3' dsDNA-dependent exonuclease. Exo will degrade linear dsDNA and generate two possible products: 1) a partially dsDNA duplex with single-stranded 3' overhangs or 2) if the dsDNA was short enough, a ssDNA whose entire complementary strand was degraded. Then, Beta protects the ssDNA created by Exo and promotes its annealing to a complementary ssDNA target in the cell (only Beta expression is required for recombineering when using ssDNA)[34,35].

Reports show the use of this strategy in several LAB: *L. lactis*[36,37], *L. reuteri*, *Lactobacillus gasseri*[34,37], *Lactobacillus casei*[38] and *Lactobacillus plantarum*[39]. The biggest challenge of using this strategy already optimized in *E. coli* may be due to specific interactions between the recombinase protein and the endogenous proteins that need to occur. Pijkeren *et al.* (2012)[37] have reported the optimization of several parameters for ssDNA recombineering in *L. lactis*, as well as identification of available RecT recombinases for use in LAB. The use of ssDNA recombineering in LAB has resulted in

specific, efficient and non-hypermutagenic mutants without the need for selection, with efficiencies of 0.3% to 20%[34]. However, ssDNA recombineering is more efficient for small modifications in the genome (point mutations, RBS/promoter substitution or premature translation termination)[37]. For large modifications, such as gene insertion or deletions, dsDNA recombineering is sometimes a better suited approach. A dsDNA recombineering strategy has been reported for *Lactobacillus plantarum* in Yang *et al.* (2015)[39] using analogues of Gam, Beta and Exo proteins (Lp_0640, Lp_0641 and Lp_0642) combined with a Cre-*lox* system. This system allows insertion and posterior removal of a selection marker allowing identification of the mutant cells[39]. However, it requires two transformation events: one to insert the marker flanked by the *lox* sites and other to introduce the Cre recombinase, and it does not result in a scarless deletion as a 45 bp long *lox72* sequence is left in the genome[5,39].

In the present work, two approaches to LAB genome editing are tested in *Lactococcus lactis* spp. *lactis* strain, LMG19460. A recombineering strategy engineered and developed by Datsenko & Wanner (2000)[40] already proven effective in *E. coli,* and promising for application in LAB is applied for the removal of an endonuclease gene (*nth*) that degrades exogenous DNA, decreasing transformation efficiency and pDNA production. An overview of this process is schematized in Figure 2.

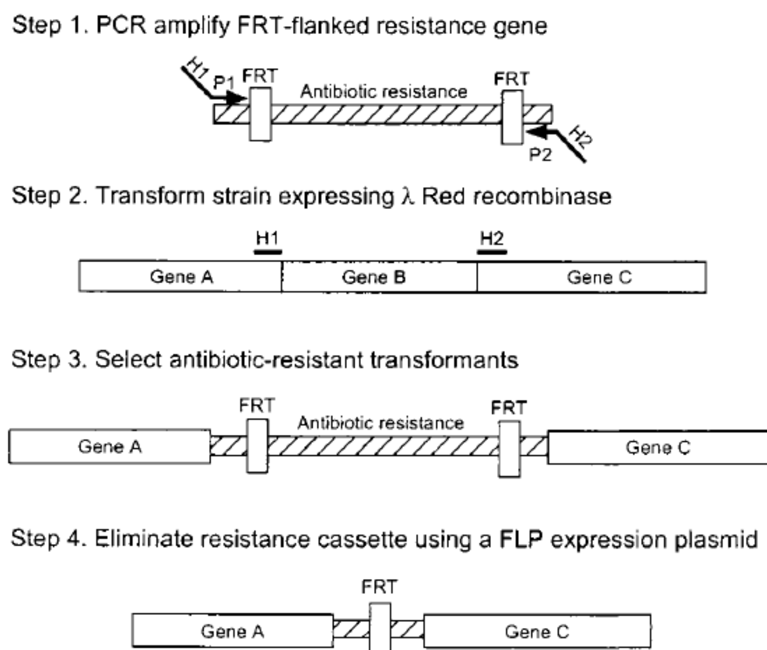


Figure 2. Overview of Datsenko & Wanner's strategy for gene disruption. H1 and H2 refer to the gene-specific homology extensions that will flank the antibiotic resistance cassette after PCR amplification. P1 and P2 refer to the priming sites that will allow PCR amplification. FRT refers to the Flippase (FLP) Recognition Target sites. Figure from Datsenko & Wanner (2000)[40].

This strategy is based on Red-phage mediated recombination of a PCR product with homology extensions targeting the gene to be disrupted. The DNA template is obtained by PCR amplification of a selectable antibiotic resistance cassette using primers with 36- to 50-nucleotide homologous arms, that will create gene-specific homology regions flanking the cassette. The primers must be designed according to the target gene. The antibiotic cassette is flanked by flippase recognition target (FRT) sites that will allow posterior removal of the selection marker by use of FLP-FRT recombination.

After the PCR product is generated, it is introduced in bacteria carrying a Red helper plasmid. This plasmid has a temperature-sensitive origin of replication and encodes for Gam, Beta and Exo λ-Red phage proteins, under control of a L-arabinose inducible P_{araB} promotor. When L-arabinose is added to the growth medium of transformed bacteria, the recombineering proteins will be expressed and mediate recombination of the homologous arms of the PCR product (DNA template) into the target site in the chromosome. These bacteria will have now the antibiotic resistance gene flanked by inverted *FRT* sites in the chromosome, instead of the original gene sequence.

Finally, the cells are transformed with a plasmid encoding a flippase (FLP), a protein derived from a *Saccharomyces cerevisiae* plasmid that with FRT sites completes the system for site-directed recombination. The plasmid has a temperature-sensitive replication and, simultaneously, encodes the FLP protein under control of a temperature-inducible promotor. This means that, when temperature is set at 43°C there will be FLP expression that will mediate excision of the antibiotic resistance cassette in the genome. At the same time, the introduced plasmids will not be able to replicate at this temperature, so they will be lost, creating a selection marker-free mutant strain[40].

This strategy has been successfully reported in *L. lactis* spp. *cremoris* (MG1363 strain) to knockout the thymidylate synthase (*thyA*) gene. In this work, a chloramphenicol-resistant cassette was amplified with homology extensions of 50 nt complementary to the *thyA* gene and then introduced in cells containing the plasmid pKD46 (from Datsenko & Wanner (2000)[40], carrying the λ-Red phage recombineering proteins)[36]. Although this strategy hasn't been fully applied for modification of food-grade LAB strains, results are promising and with optimization it may be possible to create a novel engineering tool for use in lactococcal strains.

However, one of the main setbacks of using recombineering alone is that it does not allow selection of transformants unless these carry a selection marker. Although studies are trying to create auxotrophic LAB strains for use with complementation plasmids, the most common and effective selection marker is still antibiotic resistance. Antibiotic resistance is an unwanted trait in modified bacterial strains, and genome editing strategies have to be able to select the edited cells but allow removal of the selection markers[36]. This can be achieved by curing of the used plasmids but also coupling recombineering to other systems (such as Cre-*lox*, FTR-Flp or CRISPR-Cas9 technology)[5].

1.2.1.2. CRISPR-Cas based strategies

The CRISPR (clustered regularly interspaced short palindromic repeats)/Cas (CRISPR-associated protein) system is a recent technology that allows precise genome editing and gene knockout. It is an adaptive immune mechanism present in many Eubacteria and most Archaea, to target and degrade foreign DNA that enters the cell. When bacteriophages or plasmids are introduced in organisms containing CRISPR, a fragment of this DNA is incorporated (protospacer) in the CRISPR locus between its CRISPR RNA (crRNA) repeats. Then, Cas proteins are expressed and the protospacers are transcribed into pre-crRNA which in turn is cleaved and processed into mature crRNAs by Cas proteins and host factors. The mature crRNA has part of the repeat sequence for Cas recognition and a guide homologous to the target sequence of foreign DNA. The Cas protein, guided by the RNA, recognizes the target sequence and mediates its cleavage, protecting the organism from infection. Self-cleavage of

the hosts genome is avoided because most Cas proteins only cleave the foreign DNA if the target site (complementary to the crRNA) has an adjacent protospacer adjacent motif (PAM), that is not present in the CRISPR locus (as seen in Figure3A)[41].

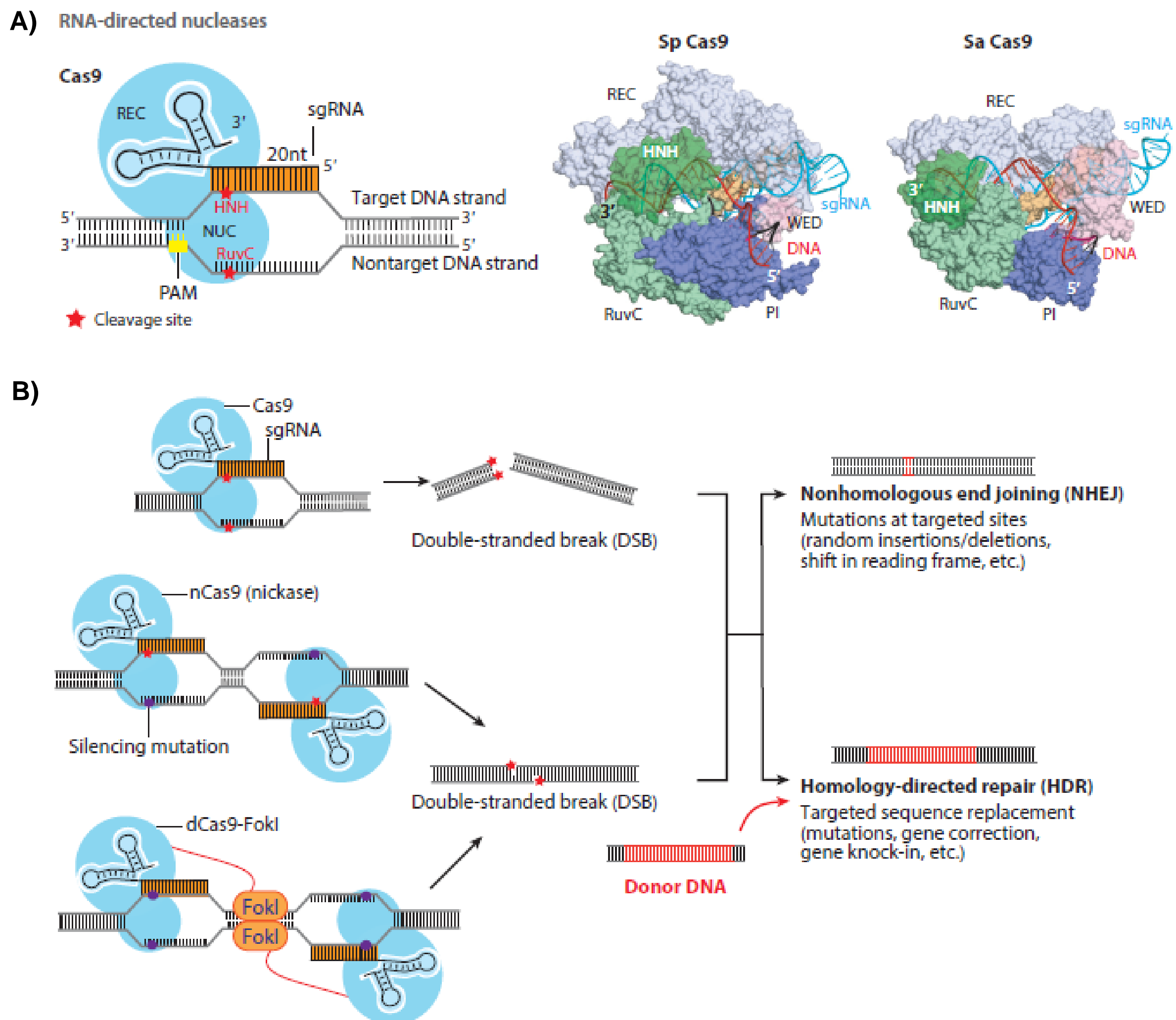


Figure 3. A) (left) Structure of the Cas9 endonuclease-sgRNA complex: Cas9 has two lobes - nuclease lobe (NUC) has two nuclease domains (HNH and RuvC), a PAM-interacting domain (PI) in conjunction with the wedge domain (WED) serves to recognize the diverse PAMs, and the recognition lobe (REC) that interacts with the sgRNA-DNA duplex; (right) Cas9 crystal structures from *Streptococcus pyogenes* (SpCas9) and *Staphylococcus aureus* (SaCas9); **B)** mode of action of Cas9-sgRNA and Cas9 variant nickase (nCas9)-sgRNA complexes, and the DNA repair mechanisms that are induced by the double-strand breaks (DBS) created: nonhomologous end joining (NHEJ) creates random insertion or deletions, homology-direct repair (HDR) needs a donor DNA for precise and controlled insertion or deletion of specific sequences[41]. Figure from Wang *et al.* (2016)[41].

The CRISPR-Cas9 system (class 1, type II) only needs an RNA-guided endonuclease, Cas9, to cleave the target DNA, and it is the most used for genome editing due to its simplicity, easy design and high efficiency. It is guided by a duplex of RNAs: the crRNA recognizes the target DNA, while noncoding trans-activating CRISPR RNA (tracrRNA) hybridizes with the crRNA repeat. This RNA duplex can be fused into a chimeric single guide RNA (sgRNA) that can be designed specifically for genome editing applications. To target a sequence in the genome, we first need to find a PAM sequence (the most

commonly used Cas9 from *Streptococcus pyogenes* (SpCas9) recognizes PAM as 5'-NGG-3') that is inside the region to be modified. The sgRNA will be designed as a sequence with approximately 20 bp that will be complementary to the 20 nucleotides adjacent to the chosen PAM sequence in the target DNA. The Cas9-sgRNA complex (Figure 3A) will identify the target sequence and cleave it, creating a double-stranded break (DSB)[41].

In eukaryotes, this will induce the DNA repair mechanisms of the cell, such as nonhomologous end joining (NHEJ), that creates random insertion or deletion mutations that can lead to gene silencing, or homology-directed repair (HDR) (Figure 3B), that can be used for replacement of a target sequence via homologous recombination guided by donor DNA leading to gene deletion, mutagenesis, insertion or gene correction[41]. Prokaryotes, however, lack the NHEJ system, thus being unable to repair the breaks in its genome without a DNA template for HDR. The inability to repair DNA breaks created by Cas9 leads to cellular death, which can be exploited as a tool for counter-selection of the mutant cells, as wild-type cells are killed due to the breaks and mutant cells will have repaired the breaks by incorporating the desired sequence in their genome (via recombineering or plasmid-based homologous recombination)[5]. This system can be an important tool in prokaryotes for creation of modified cell-factory strains for food fermentation and pharmaceutical purposes as it allows the edit to be done without the need for selection markers (antibiotic resistance is undesirable in food- and, specially, pharmaceutical-grade vectors and strains)[42].

In LAB, the native CRISPR-Cas systems are widely spread as they confer beneficial traits against phages and plasmids in the gastrointestinal tract, dairy and food fermentation environments. In *Lactococcus*, however, they are rarely present, while other genera have a high percentage of strains with encoded CRISPR-Cas systems. The use of this technology has been reported for genome editing in *L. reuteri* coupled to ssDNA recombineering[43], in *L. lactis*[44] and *L. plantarum*[45] with plasmid-based HR, for removal of mobile genetic elements (plasmids and integrative conjugative elements) without HR in *Streptococcus thermophilus*[46] and *L. lactis*[44], and for curing of a cryptic plasmid in *Leuconostoc citreum*[47]. The most recent strategy optimized for *L. lactis* combines recombineering of ssDNA and CRISPR-Cas counterselection and has been proven to allow precise point mutation, seamless deletion and insertion at efficiencies of >75% within 72 h[48].

A strategy of CRISPR-mediated ssDNA recombineering was reported by Oh & van Pijkeren (2014) in *Lactobacillus reuteri*[43] to select mutants, with 100% efficiency (previously, using only recombineering strategies the efficiency of selection was 0.3-20%[34]). The approach allowed the reproduction of point mutations using phage-derived ssDNA recombinase to integrate oligonucleotides into the genome and the efficient counter-selection of mutant cells due to the use of CRISPR technology[43].

Generally, CRISPR-Cas9 mediated strategies use plasmids with the *cas9* gene and the sgRNA sequences. In Jang *et al.* (2017), elimination of the non-curable cryptic endogenous pCB42 plasmid was made via introduction of a plasmid encoding SpCas9 and sgRNA targeting pCB42. Afterwards, the manufactured plasmid was cured through serial subculturing of the transformants in a non-selective media. Since the objective of this strategy was the degradation of an endogenous plasmid, a DNA template for homologous recombineering was not necessary[47]. For insertions and scarless deletions

and modifications in the genome, it is necessary to introduce in the cell a DNA template in addition to the plasmid coding the Cas9-sgRNA complex.

Another reported CRISPR-mediated strategy in *Lactobacillus casei*, uses a plasmid (pLCNICK) encoding a variant of Cas9 (nickase $Cas9^{D10A}$), the sgRNA and the homologous arms (targeting the gene to be modified) as DNA template for DNA repair. The nickase take the need for tightly control of Cas9 expression as it makes single stranded nicks instead of blunt double stranded breaks in the genome, that are less lethal to the cells. This method allowed precise in-frame deletions of four independent genes with 65% efficiency without the need of more than one transformation step, as well as the insertion and heterologous expression of enhanced green fluorescence protein (eGFP) gene. This simplified genome editing strategy using pLCNICK has, however, the disadvantage of having a limited deletion size of 5 kb[31].

Optimization of these strategies is still needed due to variant strain-dependent outcomes, but CRISPR-Cas mediated systems are a promising asset in LAB's engineering toolbox for creation of food- and pharmaceutical-grade improved strains. The second approach tested in the present work was created by Reisch & Prather (2015)[49] using Cas9-assisted recombineering, which allows selection of mutant cells with no scar left on its genome.

The Scarless Cas9 Assisted Recombineering (no-SCAR) strategy was designed in *E. coli* but its easy-to-use system could be optimized to make it an effective engineering tool for LAB. Moreover, this system allows easy and fast subsequent modifications, that could cover the absence of a multiplexing strategy that is missing in the LAB genome engineering toolbox[5,49].

This strategy requires three different DNA molecules: 1) the pCas9cr4 plasmid encoding the *cas9* gene under control of the P_{TET} promotor and the *tetR* gene that is constitutively expressed; 2) the pKDsgRNA plasmid that encode the sgRNA targeting the desired sequence under control of the P_{TET} promotor and the three genes that constitute the λ-Red system for recombineering, under control of the arabinose inducible P_{araB} promotor; 3) and the DNA template for recombineering (ssDNA or dsDNA oligos). As in every Cas9-sgRNA complex mediated system, the sgRNA is a sequence of approximately 20 nucleotides that target the desired gene in a region adjacent to a PAM sequence and does not show off-target activity (does not target anywhere else in the genome). The DNA donor oligos are designed with the desired mutations to be integrated in the genome, creating: point mutations, sequence deletions or insertions.

After cloning the sgRNA targeting the desired gene into the pKDsgRNA plasmid and designing the oligo with the sequence to be integrated, the cells are transformed with pCas9cr4 plasmid. Cas9 is not expressed because the TetR repressor is constantly inhibiting the P_{TET} promotor to prevent the creation of unspecific double-stranded breaks (DSB) that without a donor DNA could not be repaired, leading to cell death. Then, these are transformed with the plasmid encoding the sgRNA and the λ-Red genes. The transformed cells are selected by the antibiotic resistance the plasmids carry. Only after inducing expression of the λ-Red proteins with L-arabinose, is the oligo introduced in the cells. The induction of λ-Red proteins cannot be prolonged since the activity of Gam can be toxic to the cell. When the elements

needed for recombineering (λ-Red proteins and the DNA template) are present in the cell, the expression of Cas9 and the sgRNA are induced, and the system will integrate the oligo on the targeted site. This allows counter-selection of the wild-type cells, since the only cells that are able to grow are the ones that had the DSB repaired by the recombineering system (this process is schematized in Figure 4)[50].

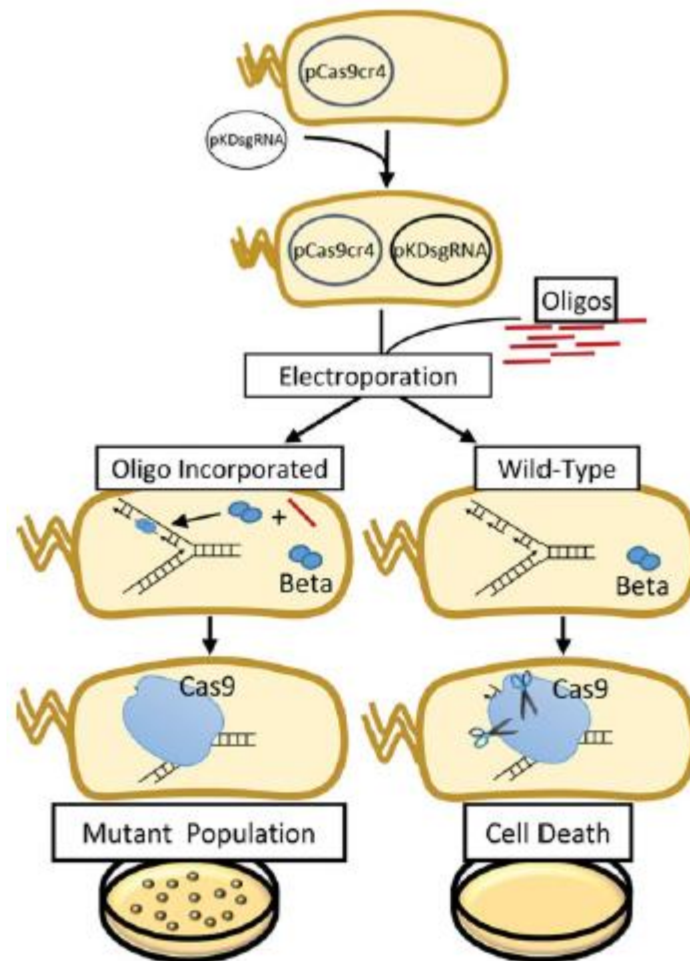


Figure 4. Schematic of the no-SCAR system process, highlighting the counter-selection of mutant cells by cell death of the wild-type. Figure from Reisch & Prather (2017)[50].

After selection, the plasmids used in the strategy have to be cured to erase any selection markers from the inside of the cell. For pKDsgRNA plasmids, the origin of replication is temperature sensitive, thus, it does not replicate at 37°C. The pCas9cr4 plasmid, on the other hand, does not encode any trait that can be exploited for curing. The authors suggest the use of a pKDsgRNA plasmid targeting a feature in this plasmid, so that the Cas9 can recognize and degrade it. So, the pKDsgRNA-p15 plasmids targets the p15A origin of replication in pCas9cr4 and allows curing of this plasmid with a final step of transformation[49]. For subsequent mutations, the pKDsgRNA-xxx plasmid can be cured at 37°C, and then a new plasmid with a different sgRNA can be introduced along with the new DNA donor oligo. This could allow fast and easy modification of the genome and fast creation of optimized strains.

In LAB there have been several reports on the use of CRISPR-Cas9 mediated recombineering with successful results. The strategy here described is a promising method for application in LAB, however, optimization of some steps is still necessary so that every element of the system is functional in the cell and strain optimization is possible. The most evident setback, however, is that one of the plasmids (pCas9cr4) has an origin of replication that does not allow replication in LAB. It is thus necessary to turn the plasmid into a shuttle vector, ideally, with a broad range LAB origin of replication. The common plasmids used in optimization of lactococcal strains for DNA or mucosal vaccination are promising candidates (pWV01- , pSH71- or pAMβ1-based replicons) as reports show their efficiency and broad host range[14,51,52]. To turn pCas9cr4 into a shuttle-vector, the Gram-positive origin of replication must not interfere with replication from p15A. This makes the pAMβ1 origin of replication the best option since it will not compete with the replication machinery while inside *E. coli* but allows replication in a wide range of Gram-positive hosts[51].

Furthermore, it is necessary to optimize antibiotic concentration for selection of the transformed cells and induction of expression from the P_{TET} promotor. Taking into consideration that different strains may have different resistances some antibiotics may not be the best choice for application in different LAB species. However, with the right optimization, this strategy poses a promising tool for fast and efficient genome editing in LAB, especially in lactococcal strains. So far, all studies regarding genome engineering in LAB only allow one modification at a time. Targeting of multiple genes at a time (multiplexing) could be a necessity in many applications. The ability to rapidly target subsequent genes makes this strategy one of the most auspicious for fast strain engineering and creation of optimized strains.

2. Background and objectives

Lactic acid bacteria are a promising tool for food industries and new biomedical applications. The historically safe use of these bacteria in food industries and their contribution to the healthy microflora of human mucosal surfaces has granted them a GRAS status. Their ability to colonize mucosal tissues and survive the passage through the GIT, allied with the healthy and safe use they demonstrate, make them advantageous for use in novel biomedical strategies, such as cell-factories or live delivery vectors of pharmaceutical-grade proteins and pDNA.

These group of bacteria could provide a more effective and precise delivery of pDNA and recombinant proteins into mammalian cells, through mucosal vaccination, with enhanced potency and lesser side effects than systemic and attenuated vaccines. Another application is the use of these bacteria as cell-factories for large-scale production of pDNA for therapeutic use (such as in gene therapy). However, a high yielding GRAS bacterial producer of pDNA and proteins has yet to be optimized.

The aim of this work is to start the optimization of a plasmid-free, non-pathogenic, non-sporulating, non-motile, with GRAS status strain of *L. lactis*. The *L. lactis* LMG19640 strain is an auspicious choice for a safe and effective cell-factory and/or live delivery vector of pharmaceutical-grade proteins and pDNA. Firstly, however, the strain needs to be engineered to have a high yield of pDNA production. The main setback of this strain is the presence of some genes that hinder pDNA production or enhance degradation of exogenous DNA.

In this work, two different genetic engineering strategies were attempted for the knockout of the *nth* gene, which encodes for endonuclease III, responsible for exogenous DNA degradation. The first strategy is based on the λ-Red recombineering system, using the λ-Red proteins to incorporate a kanamycin resistance cassette in the target site in the genome, removing the desired gene and subsequently, the kan-cassette is removed, leaving a small scar in the genome. The second strategy uses recombineering coupled to a CRISPR-Cas9 system for counter-selection of mutants – the recombineering proteins will integrate a specifically designed oligo into the target site in the genome and the CRISPR-Cas system will target the genomes in which the oligo was not incorporated, leading to death of the wild-type cells. These strategies were designed in *E. coli*, thus needing some optimization steps for effective use in Gram-positive bacteria, that are described throughout this work.

3. Materials and Methods

3.1. Bacterial strains and plasmids

The characteristics of the strains and plasmids used throughout this work are described in Table 1. All molecular cloning steps and plasmid production was done in *E. coli* DH5α. The pTRKH3 plasmid (shuttle vector) was used for transformation controls when needed and as donor of pAMβ1 origin of replication and erythromycin resistance marker (EryR) for the modified pCas9cr4 plasmid. Genomic sequence for *nth* gene (NC_002662) was retrieved from *L. lactis* spp. *lactis* IL1403 (already sequenced and similar to *L. lactis* spp. *lactis* LMG19460).

Table 1. Main characteristics of the bacterial strains and plasmids used in the present work, for the gene knockout strategies by Datsenko & Wanner (2000)[40] and Reisch & Prather (2015)[49].

Strain	Characteristics	Source
***Escherichia coli* DH5α**	F- Φ80*lacZ*ΔM15 Δ(*lacZYA-argF*) U169 *recA1 endA1 hsdR17* (rk-, mk+) *phoA sup*E44 λ-*thi*-1 *gyr*A96 *rel*A1	Invitrogen
***Lactococcus lactis* LMG 19460**	Wild-type, plasmid free strain[12]	LMG/BCCM Culture Collection, Belgium
Plasmid	**Characteristics**	**Source**
pTRKH3	p15A *ori*, pAMβ1 *ori*, Tet^R, Ery^R	LMBP 4462/BCCM Culture Collection, Belgium
pKD46	oriR101 with repA101ts, Gam-beta-exo proteins under the control of arabinose inducible promoter P_{araB}, Amp^R	Datsenko & Wanner (2000)[40]
pKD13	oriR6Kgamma, Kan^R cassette flanked by FRT sites, Amp^R	
pCP20	oriR101 with repA101ts, FLP^+, λ $cI857^+$, λ pR Rep^{ts}, Cm^R, Amp^R	Cherepanov & Wackernagel (1995)[53]
pCas9cr4	p15A ori, *cas9* expressed under control of the P_{TET} promoter, *tetR* constitutively expressed, Cm^R	Reisch & Prather (2015)[49]
pKDsgRNA – p15	oriR101 with repA101ts, sgRNA under control of the P_{TET} promoter, Gam-beta-exo proteins under the control of arabinose inducible P_{araB}, $Spec^R$	
pKDsgRNA – nth	pKDsgRNA – p15 derivative with specific sgRNA sequence targeting the *nth* gene	This study
pCas9cr4_pAMβ1_ery	pCas9cr4 derivative with pAMβ1 origin of replication and Ery^R	This study

Ery^R, Amp^R, Kan^R, Cm^R and $Spec^R$: resistance marker to erythromycin, ampicillin, kanamycin, chloramphenicol and spectinomycin, respectively.

3.2. Media and growth conditions

Growth of *E. coli* DH5α was performed in 20 g/L of Luria-Bertani (LB) broth (Nzytech) supplemented with the respective antibiotics for selection of pTRKH3, pCas9cr4 and pKD13 plasmids: 500 µg/mL erythromycin (Sigma-Aldrich), 50 µg/mL chloramphenicol (Sigma-Aldrich) and 25 µg/mL kanamycin

(Sigma-Aldrich), at 37°C, 250 rpm. For *E. coli* DH5α with pKDsgRNA – p15, pKD46 and pCP20 plasmids and derivatives, growth was performed in the same medium supplemented with 50 µg/mL spectinomycin (Sigma-Aldrich),100 µg/mL ampicillin (Sigma-Aldrich) and 25 µg/mL chloramphenicol (Sigma-Aldrich), respectively, at 30°C, 100 rpm. *L. lactis* LMG19460 standard growth was done in M-17 (pH 7.0, Fluka) supplemented with 20 g/L glucose monohydrate (Labchem) and the respective antibiotics for selection (except for cells containing the kanamycin resistance cassette, in which neomycin was used), at 30°C, 100 rpm. Antibiotic concentration for selection in the *L. lactis* strain was varied and assessed throughout the laboratory work. Cell banks for storage were made with 20% (v/v) glycerol and kept at -80°C.

According to some experiments throughout this work, a need for assessment of *L. lactis* LMG19460 growth in other media was necessary. Thus, MRS media, LB broth and Elliker liquid medium (composed of tryptone 20 g/L (BD Biosciences), glucose 5 g/L (Sigma-Aldrich), yeast extract 5 g/L (Liofilchem), NaCl 4 g/L, sodium acetate 1.5 g/L, ascorbic acid 0.5 g/L at pH of 6.8), were tested, with and without added 0.1% L-arabinose.

When in solid media, *E. coli* DH5α was grown in LB agar (2%) plates supplemented with the appropriate antibiotic, at 30/37°C (according to the presence of thermosensitive plasmids). *L. lactis* LMG19460 was grown in solid regeneration medium[54] with the following composition: 10 g/L tryptone (BD Biosciences), 5 g/L yeast extract (Liofilchem), 200 g/L sucrose (Fisher Scientific), 10 g/L glucose (Sigma-Aldrich), 25 g/L gelatin (Merck), 15 g/L agar (JMV Pereira), 2.5 mM $MgCl_2.6H_2O$ (Fagron), 2.5 mM $CaCl_2$ (V. Reis), supplemented with the appropriate antibiotic and incubated at 30°C.

3.3. Cell competence and transformation

3.3.1. Chemically competent *E. coli* DH5α cells[55]

To prepare chemically competent *E. coli* DH5α a pre-inoculum was grown overnight in 5 mL of LB broth, and then used to inoculate a 100 mL shake flask with 20 mL LB broth (initial optical density at 600nm (OD_{600nm}) = 0.1). Cells were grown at 37°C, 250 rpm, until reaching an OD_{600nm} of 1. The grown cells were centrifuged (1,000 g, 10 min, 4°C) and the pellets resuspend in 0.1 volumes (2 mL) of chilled TSS medium (20 g/L LB, 5% DMSO, 50 mM $MgCl_2$, 10% PEG 8000 (w/v), pH=6.5). Aliquots of 100 µL were made and kept on ice for 10 min, then stored at -80°C.

Chemically competent cells were transformed by heat shock with 100 ng of the desired plasmid. According to each experiment, 100 µL aliquots were incubated with the appropriate amount of pDNA for 30 min on ice. The mixture was submitted to 42°C for 1 min, immediately incubated on ice for 2 min, and resuspended in 900 µL of LB broth. After 1h incubation at 37°C, 250 rpm, the cells were plated on LB agar supplemented with the appropriate antibiotic and incubated at 30 or 37°C (depending on the plasmid).

To confirm the transformation of *E. coli* DH5α chemically competent cells, the obtained colonies were inoculated in LB with the appropriate antibiotic and incubated overnight at 30 or 37°C. The culture was then centrifuged (6,000 g, 3 min, 4°C). and the pellet was used for plasmid purification using High Pure Plasmid Isolation Kit (Roche). The resulting pDNA was observed in a 1% agarose gel.

3.3.2. Electrocompetent *E. coli* DH5α cells[56]

To prepare electrocompetent *E. coli* DH5α a pre-inoculum was grown overnight in 5 mL of LB broth, and then used to inoculate a 1 L shake flask with 250 mL LB broth (initial OD_{600nm}=0.1). Cells were grown at 37°C, 250 rpm, until reaching an OD_{600nm}=0.5-0.7. Following steps were made keeping the cells at minimum temperature possible (using chilled solutions kept on ice). The grown cells were centrifuged (5,000 rpm, 10 min, 4°C) and washed with 1 volume of 10% glycerol, three times. Lastly, the final pellets were resuspended in 1 mL of 10% glycerol, and parted in 50 µL aliquots, that were then stored at -80°C.

Electrocompetent *E. coli* DH5a cells were transformed by electroporation. According to each experiment, the appropriate amount of pDNA was added to the 50 µL aliquots. The mixture was transferred to 2 mm chilled electroporation cuvettes and subjected to one pulse of 2,000 V for 8 ms using an electroporator (Gene Pulser Electroporator, BioRad). The cells were resuspended in 950 µL of warm LB broth, and incubated for 1h at 37°C, 250 rpm. Finally, the cells were plated in LB agar supplemented with the appropriate antibiotic and incubated at 37°C.

To confirm the transformation of electrocompetent *E. coli* DH5α cells, the obtained colonies were inoculated in LB with the appropriate antibiotic and incubated overnight at 37°C. The culture was then centrifuged (6,000 g, 3 min, 4°C). and the pellet was used for plasmid purification using High Pure Plasmid Isolation Kit (Roche). The resulting pDNA was observed in a 1% agarose gel.

3.3.3. Electrocompetent *L. lactis* LMG19460 cells

3.3.3.1. Holo & Nes (1989)[54] glycine protocol

Electrocompetent *L. lactis* LMG19460 cells were prepared using an adapted and optimized protocol from Holo & Nes (1989)[54]. A pre-inoculum was grown overnight in 5 mL of M-17 supplemented with 20 g/L glucose (GM-17), at 30°C, 100 rpm, and then used to inoculate a 100 mL shake flask with 75 mL M-17 supplemented with 0.5% glucose (initial OD_{600nm}=0.1). Cells were grown at 30°C, 100 rpm, until reaching an OD_{600nm}=0.5-0.8. A 100-fold dilution (750 µL) of the grown cells was made onto 75 mL of fresh GM-17 medium supplemented with 0.5 M sucrose (SGM-17) and 1-2% glycine, and the cells were grown at 30°C, 100 rpm. When the cells reached an OD_{600nm}=2-2.5, they were centrifuged (6,000 g, 3 min, 4°C) and washed twice with 1 mL of ice-cold washing solution (sucrose 0.5 M and glycerol 10% (v/v)). Lastly, the cells were resuspended in 1/100 of the initial culture volume of washing solution, parted into 80 µL aliquots, and then stored at -80°C.

For electrotransformation of *L. lactis* LMG19460 cells, according to each experiment, the appropriate amount of pDNA was added to 40 µL aliquots containing approximately $1x10^9$ electrocompetent cells (1 OD unit at 600 nm is equivalent to $7x10^8$ cells/mL)[57]. The mixture was transferred to 1 mm electroporation cuvettes and subjected to 2-3 pulses (variable according to experiments) of 1,000 V for 7-9 ms using an electroporator (Gene Pulser Electroporator, BioRad).

Immediately after electroporation, 960 µL of ice-cold recovery medium (SGM-17 supplemented with 20 mM $MgCl_2$ and 2 mM $CaCl_2$) was added and the mixture was incubated on ice for 5 min. The cells

were incubated for 3h at 30°C without agitation. Then they were centrifuged (1 min, 6,000 g) and used to inoculate 5 mL of GM-17 supplemented with a sub-lethal concentration of the appropriate antibiotic. The cells were grown at 30°C without agitation, overnight. Finally, the cells were plated in solid regeneration medium (as described in section 3.2) and grown at 30°C.

The resulting colonies were grown overnight at 30°C in GM-17 with the appropriate antibiotic and then tested for plasmid presence by purification using Nucleospin Plasmid Kit (Macherey-Nagel). The resulting pDNA was run and observed in a 1% agarose gel.

3.3.3.2. Palomino et al.[58] high salt concentration protocol

Electrocompetent *L. lactis* were prepared using high NaCl concentrations in M-17 or MRS medium, when low concentration of sugar was necessary. Cells were grown overnight in 5 mL of liquid media, at 30ºC, 100 rpm, and then used to inoculate 100 mL of the liquid medium supplemented with 0.7 M NaCl, with an initial OD of 0.1. After an overnight growth, the culture was centrifuged at 6,000g, 3 min, 4ºC and washed 3 times in ice cold water. They were resuspended in a 20% glycerol solution, distributed in 100 µL aliquot and stored at -80ºC.

3.4. Confirmation of *L. lactis* LMG19460 identity

Contaminations and electrocompetence were tested by transforming the cells (as described in section 3.3.3.1.) with 100 ng of the pTRKH3 plasmid and using PCR-grade water for negative control. The transformed cells were plated in solid regeneration medium[54] supplemented with 5 µg/mL of erythromycin (lethal for wild-type *L. lactis* LMG19460 cells).

To confirm the identity of the cells used throughout this study a molecular protocol was used, based in Salbi *et al.* (2014)[59]. The *hisG* gene (NC_002662, *L. lactis* spp. *lactis* IL1403) allows for identification of *Lactococcus* species, if the cells tested belong to an *L. lactis* strain, PCR amplification with the primers in Table 2, results in a 933 bp fragment. When needed, cells were tested from their genomic DNA or by colony PCR.

Table 2. Primers used for amplification of *hisG* gene, specific for identification of *Lactococcus* species.

Primer	Sequence (5' - 3')	Product size
hisG_F	CTTCGTTATGATTTTACA	933 bp
hisG_R	CAATATCAACAATTCCAT	

Extraction of genomic DNA was performed with Wizard Genomic DNA Purification Kit (Promega). When genomic DNA (gDNA) was successfully extracted, a PCR reaction was set using the KOD Hot Start DNA Polymerase kit (Novagen) with 100 ng of gDNA, 0.02 U/µL of KOD DNA Polymerase, 2.3 mM of MgSO4, 1x buffer, 0.2 mM of dNTPs, 0.3 µM of each primer and completed with PCR-grade water to a final volume of 25 µL. The cycling conditions were: initial denaturation for 3 min at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 41.5°C and 1 min at 70°C.

For confirmation via colony PCR, the tested colonies were resuspended in 40 µL sterile PCR-grade water, 10 µL (10^7 cells) were used in the PCR reaction, while 20 µL of this was used for inoculation of 5

mL GM-17 medium if results turned out positive and new master banks were needed. The PCR reaction was performed with KOD Hot Start DNA Polymerase kit (Novagen) with the same composition already described in this section, whereas cycling conditions were the same except with a 10 min at 95°C for cell lysis and initial denaturation.

3.5. Condition optimization

3.5.1. Antibiotic concentration

To find the ideal antibiotic concentration for selection of the plasmids for the *L. lactis* LMG19460 strain, electrocompetent cells made with 1% and 2% glycine were grown on solid regeneration medium[54] (as described in section 3.2.) supplemented with different antibiotic concentrations (Table 3). Analysis was done in a range of concentrations comprising values registered in literature[12,60,61,62] and previous works with these bacteria, and as control, a replica was done with no addition of antibiotic.

Table 3. Range of concentrations analysed to find optimal concentration for selection by each antibiotic.

Antibiotic	Tested concentrations (µg/mL)
Chloramphenicol	1, 1.35, 1.5, 2, 2.35, 5, 10, 15, 25, 50, 75, 500, 1000
Spectinomycin	25, 50, 75, 100, 250, 500, 750, 1000
Ampicillin	1, 1.25, 1.5, 2, 2.5, 5, 10, 15, 20, 25, 50, 100, 150
Neomycin	1000, 1500, 1750, 2000

Later experiments were made using the assessed optimal concentration, however, some optimizations were needed according to the procedure (that are specified in the corresponding sections).

3.5.2. Growth in anhydrotetracycline (aTc)

Growth limits in anhydrotetracycline (aTc) were tested for following induction of the *Cas9* gene and expression of the sgRNA in Reisch & Prather (2015) protocol[49]. *L. lactis* LMG19460 cells made with 1% glycine were grown in solid regeneration medium[54] supplemented with 0 (control), 50, 100 (concentration described as inductive in the protocol) and 200 µg/mL of aTc.

3.6. Reisch & Prather (2015)[49] strategy

The Scarless Cas9 Assisted Recombineering (no-SCAR) strategy requires: 1) the pCas9cr4 plasmid encoding the *cas9* gene under control of the P_{TET} promotor and the tetR gene that is constitutively expressed (Figure 5A); 2) the pKDsgRNA plasmid that encodes the sgRNA targeting the *nth* gene under control of the P_{TET} promotor and the three genes that constitute the λ-red system for recombineering, under control of the arabinose inducible P_{araB} promotor (Figure 5B); 3) a DNA template (oligo) for recombineering-mediated integration in the genome after the Cas9-sgRNA complex creates double-stranded breaks (DBS) within the *nth* gene. After transformation of the cells with these three DNA molecules and induction of the encoded genes, the *nth* gene should be eliminated from the genome by integration of the oligo, and only mutant cells will be obtained (counter-selection by the CRISPR-system

due to inability of the wild-type cells to repair the DSB). In the following section the steps taken in the process of adjustment of the protocol to the *L. lactis* LMG19460 strain are shown.

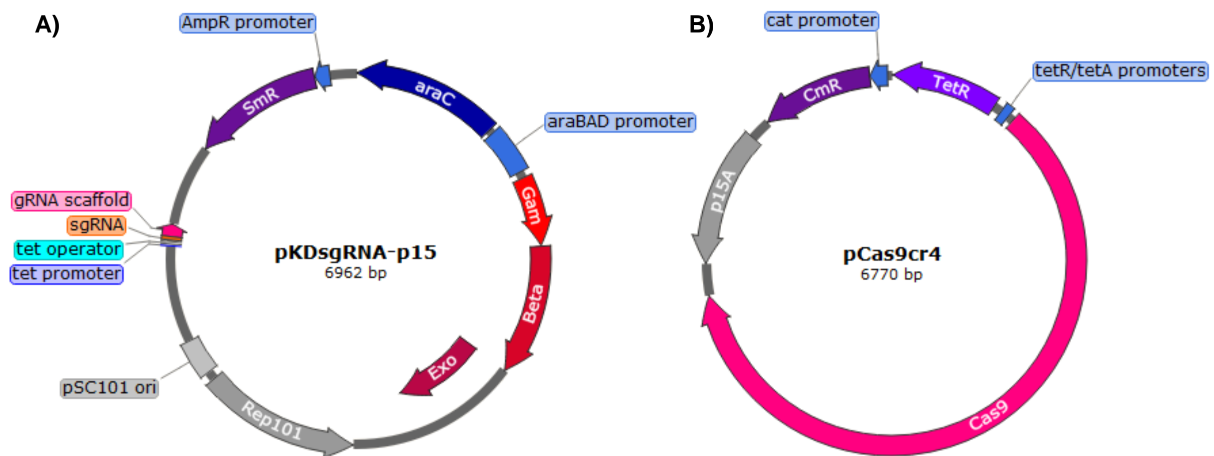


Figure 5. Map of the Reisch & Prather (2015) [49] original plasmids: **1)** pKDsgRNA-p15, **2)** pCas9cr4.

3.6.1. *In silico* design of the DNA template for DSB repair following the Reisch & Prather (2017)[50] protocol

The DNA template (ssDNA donor oligo) to be integrated in the genome was designed with homology to the 40 bp upstream and 40 bp downstream flanking the *nth* gene in the genome, using the APE[63] and SnapGene[8] softwares. Assess of secondary structures was made using the online software mfold (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form) using the default parameters[64].

3.6.2. Cloning of sgRNA targeting *nth* gene in pKDsgRNA-p15

The guide RNA was selected to target the *nth* gene using an online software (CRISPR MultiTargeter[65]). The option with the highest score from the possible 20 bp sequences immediately upstream a 5'-NGG-3' PAM sequence was chosen. Potential off-target sites were verified using the online software Cas-OFFinder[66].

To clone the sgRNA into the pKDsgRNA-p15 plasmid a Circular Polymerase Extension Cloning (CPEC) strategy was used, following the Reisch & Prather (2017)[50] protocol. CPEC requires linear DNA fragments produced by PCR that contain short overlapping sequences on both ends. For this, the pKDsgRNA-p15 plasmid was amplified using the primers in Table 4.

Table 4. Primers used for cloning of sgRNA targeting the nth gene by CPEC (the guide RNA is shown in **bold**), and for sequencing for confirmation of cloning.

Primer	Sequence (5' – 3')
sgRNA_nth_F	**gcagaagcctacggaattcc**GTTTTAGAGCTGTGAAAACAGC
sgRNA_nth_R	**ggaattccgtaggcttctgc**GTGCTCAGTATCTCTATCACTGA
pKDsgRNA-frag2fwd	CCAATTGTCCATATTGCATCA
pKDsgRNA-frag1rev	TCGAGCTCTAAGGAGGTTATAAA
sgRNA_conf	AGCTTTCGCTAAGGATGATTT

The pKDsgRNA-p15 plasmid was amplified by PCR using the sgRNA_nth_F and pKDsgRNA-frag1rev primers, yielding a 2,868 bp fragment, and the sgRNA_nth_R and pKDsgRNA-frag2fwd primers giving a 4,434 bp fragment. These fragments have a 276 bp overlap in one end and the 20 bp sgRNA sequence overlapping in the other. The PCR amplifications was done using KOD Hot Start DNA Polymerase kit (Novagen), by mixing 10ng of pKDsgRNA-p15 plasmid, 0.02 U/μL of KOD DNA Polymerase, 1 mM of $MgSO_4$, 1x buffer, 0.2 mM of dNTPs, 0.3 μM of each primer and completed with PCR-grade water to a final volume of 25 μL. The amplification conditions were: initial denaturation for 3 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C and 5 min at 70°C. After amplification, the template was digested with 1 μL of DpnI (Promega), at 37°C for 2 hours and run on a 1% agarose gel. The specific bands were excised from the gel and purified with NZYGelPure kit (Nzytech). The purified fragments were concentrated using a DNA SpeedVac Concentrator and resuspended in 5 μl of PCR-grade water. To perform the CPEC cloning, the fragments were mixed and with KOD Hot Start DNA Polymerase kit (Novagen) a PCR reaction was set up with the following conditions: initial denaturation for 3 min at 95°C, followed by 15 cycles of 1 min at 95°C, 1 min at 57°C and 5 min at 70°C. Lastly, 5 μl and 20 μl of the final construction were used to transform (as described in section 3.3.1.) chemically competent *E. coli* DH5α. The cells were plated in LB agar supplemented with 50 μg/mL of spectinomycin and incubated at 30°C.

The resulting colonies from each transformation (with 5 or 20 μl of the final PCR mixture) were isolated and grown in LB medium with 50 μg/mL spectinomycin, at 30°C, 100rpms, overnight. Plasmid DNA from each colony was purified using High Pure Plasmid Isolation Kit (Roche), measured using Nanodrop Spectrophotometer (Nanovue Plus, GE) and concentrated using DNA SpeedVac Concentrator. The purified plasmids were sent to Stabvida for sequencing with the confirmation primer sgRNA_conf. The colony with the correct construction (pKDsgRNA-nth) was stored in 100 μl aliquots with 20% glycerol at -80°C.

3.6.3. Cloning of pAMβ1 origin of replication and erythromycin resistance gene in pCas9cr4 plasmid

Cloning of the pAMβ1 origin of replication and the erythromycin resistance gene (*erm*) in pCas9cr4 plasmid was attempted by DNA ligation or Gibson Assembly. The experiments were done in parallel to add only the pAMβ1 ori or a fragment containing both the origin of replication and the *erm* gene. The fragments for both approaches were obtained by PCR amplification with the primers in Table 5. The primers were designed using APE[63] and Snapgene[8] and NEB Builder Assembly Tool[67], and synthesized by Stabvida.

Linearization of pCas9cr4 and simultaneous addition of SgsI and BsrGI enzyme restriction sites and Gibson Assembly overlaps (between Cas9 gene and p15 origin of replication) was done by PCR amplification with the primers pCas9cr4_assembly_F/R, using the KOD Hot Start DNA Polymerase kit (Novagen). The reaction was made with 10 ng of pCas9cr4 plasmid, 0.02 U/μL of KOD DNA Polymerase, 2.3 mM of MgSO4, 1x buffer, 0.2 mM of dNTPs, 0.3 μM of each primer and completed with

PCR-grade water to a final volume of 25 µL. Cycling conditions were as follows: initial denaturation for 3 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 60°C and 7 min at 70°C.

Table 5. Primers used for amplification and linearization of pCas9cr4 plasmid (vector) and amplification of pAMβ1 and pAMβ1 + *erm* fragments (insert) for molecular cloning with restriction enzymes and Gibson assembly. CAPITAL LETTERS: annealing regions; dark grey underlined: overlap region for Gibson assembly; **bold**: restriction target site for SgsI (**ggcgcgcc**) or BsrGI (**tgtaca**); light grey: protection nucleotides for efficient enzyme digestion.

Primer	Sequence (5' – 3')	Product size
pCas9cr4_assembly_F	aat***ggcgcgcc***TGCTTGGATTCTCACCAATAAAAAAC	6,770 bp
pCas9cr4_assembly_R	cgg***tgtaca***CTAGTAACAACTTATATCGTATGGG	
pAMβ1_assembly_F	acgatataagttgttactag***tgtaca***ccgCTAGCGCTCTTATCATGG	3,518 bp
pAMβ1_assembly_R	tattggtgagaatccaagca***ggcgcgcc***attGAATTCTATTTAATCACTTTGACTAG	
pAMβ1_ery_assembly_F	acgatataagttgttactag***tgtaca***ccgGATTACATGAACAAAAATATAAAATATTCTC	4,377 bp
pAMβ1_ery_assembly_R	tattggtgagaatccaagca***ggcgcgcc***attGCTCATCCGGAATTCTATTTAATC	

Amplification of pAMβ1 origin of replication and simultaneous addition of flanking SgsI and BsrGI enzyme restriction sites was done by PCR amplification of the pTRKH3 plasmid, with the primers pAMβ1_assembly_F/R, using KOD Hot Start DNA Polymerase kit (Novagen). The mixture and the cycling conditions were as described above as amplification of both fragments was done simultaneously. The pAMβ1 + *erm* fragment was amplified from pTRKH3 in the same conditions, using the primers pAMβ1_ery_assembly_F/R. The PCR reactions were digested for 2 h with 1 µL of DpnI (Thermo Fisher Scientific) and then column-purified using NZYGelPure – PCR clean-up (Nzytech).

To make compatible ends for molecular cloning, 3 µg of the resulting fragments were digested using the mixture: 0.5 µL of SgsI and BsrGI (Thermo Fisher Scientific), buffer Tango 1x (Thermo Fisher Scientific), completed with PCR-grade water to a final volume of 40 µL. The digestion reactions were incubated at 37°C for 3 hours, and then 5 µL of reaction was run in a 1% agarose gel to confirm the fragments size. When no unspecific bands occurred, the fragments were column purified using NZYGelPure – PCR clean-up (Nzytech). If unspecific bands appeared, the correct bands were excised from the gel and purified using NZYGelPure (Nzytech). All DNA concentrations were quantified using a Nanodrop Spectrophotometer (Nanovue Plus, GE).

Ligation of the two fragments was done with 100 ng of the vector and considering a 3:1 insert/vector molar ratio, according to the equation:

$$ng\ insert = \frac{mg\ vector\ \times size\ (kb) insert}{size\ (kb) vector} \times molar\ ratio\ {}^{insert}/_{vector}$$

With 100 ng of pCas9cr4 linearized plasmid (vector) to 155.4 ng of pAMβ1 fragment or 193.4 ng of pAMβ1+erm (insert).

The ligation mixture of: 2 μL T4 DNA ligase 3 U/μL (Promega) and 2 μL T4 ligase buffer x10 (300 mM Tris-HCl (pH 7.8), 100 mM MgCl2, 100 mM DTT, 10 mM ATP) completed with PCR-grade water to a final volume of 20 μL, was incubated with the vector and insert for 3 hours at room temperature. After 3 hours of incubation, chemically competent *E. coli* DH5α cells were transformed (as described in section 3.3.1.) with 10 μL of the mixture. The remaining 10 μL were kept overnight at 4°C and used for transformation of another aliquot of chemically competent *E. coli* DH5α cells.

The ligation procedure previously described was repeated with 1:1 (100 ng of pCas9cr4 linearized plasmid to 52.5 ng of pAMβ1 and 64.5 ng of pAMβ1+erm) and 1:2 (100 ng of pCas9cr4 to 105 ng of pAMβ1 and 129 ng of pAMβ1+erm) vector:insert molar ratio. The overnight ligation at 4°C was also optimized, to allow the samples to go through all temperatures from room temperature until 4°C. A thermocycler programed to decrease 0.2°C for each 10 min was used, starting at 19°C, the reaction was used for transformation of chemically competent *E. coli* DH5α cells. Further repetitions were done with the same strategy but using electrocompetent *E. coli* DH5α cells (as described in section 3.3.2.).

Simultaneously, cloning was attempted by Gibson Assembly using NEBuilder HiFi DNA Assembly Cloning Kit (New England Biolabs). Fragments were amplified, and column purified as in the restriction enzyme approach but did not undergo digestion. Assembly of the two fragments was attempted with 60 or 100 ng of the vector and considering a vector:insert mass ratio of 1:1, 1:2 and 1:3. The amount of each fragment was calculated using the following equation to obtain a maximum of 0.3 ρmol of DNA in each reaction:

$$\rho mol\ = \frac{(weight\ in\ ng)\ x\ 1{,}000}{(base\ pairs\ x\ 650\ daltons)}$$

The reaction mixture of 10 μL of NEBuilder HiFi DNA Assembly Master Mix (composed of three enzymes - T5 exonuclease, a DNA polymerase with proofreading abilities and a DNA ligase - and a reaction buffer with PEG-8000, Tris-HCl pH 7.5, $MgCl_2$, DTT, dNTPs and NAD[68]) and the necessary volume of each fragment completed with PCR-grade water to a final volume of 20 μL, was incubated at 50ºC for 1 hour and 3 hours. After incubation, 10 μL of the reaction mixture were used to transform chemically competent *E. coli* DH5α.

In both approaches, the resulting colonies were grown in liquid media with chloramphenicol (for cloning of pAMβ1 ori) and erythromycin (for cloning of pAMβ1 + erm fragment) and used for plasmid purification using High Pure Plasmid Isolation Kit (Roche). The resulting pDNA was digested with 0.5 μL of SgsI

and BsrGI (Thermo Fisher Scientific), buffer Tango 1x (Thermo Fisher Scientific), completed with PCR-grade water to a final volume of 20 µL, incubated at 37°C for 3h and the resulting fragments were run and observed in a 1% agarose gel. The plasmid samples showing a correct band pattern in the electrophoresis gel, were sent to Stabvida for sequencing. All constructions in this section were sequenced with the primers in Table 6.

Table 6. Sequencing primers for confirmation of pCas9cr4 plasmid cloning.

Primer	Sequence (5' – 3')	Obs.
pCas9_conf_new	CTCTTCAAATGTAGCACCT	targets *erm* gene (results in complete sequence, most times)
pAMβ1_conf_beg	CGTTACTAAAGGGAATGTAGA	targets beginning of pAMβ1 sequence
pAMβ1_conf_end	GGATTTGTTCAGAACGCTCG	targets end of pAMβ1 sequence (reverse)

Simultaneously, electrocompetent *L. lactis* LMG19460 cells were transformed with 100 ng of the obtained plasmid, with 3 to 5 electric pulses, as described in section 3.3.3.1. Isolated colonies that grew in GM-17 medium with antibiotic were plasmid-purified using Nucleospin Plasmid Kit (Macherey-Nagel). The resulting pDNA was double digested with SgsI and BsrGI in buffer Tango 1x (Thermo Fisher Scientific) at 37°C for 3h, and was, later, linearized with with 0.5 µL BamHI in 1x buffer E (Promega), BglII in 1x buffer D (Promega) or Alw44I in Tango 2x (Thermo Fisher Scientific). for 2h at 37°C. The digested products were run in an agarose electrophoresis gel and a pDNA sample was sent to Stabvida for sequencing.

3.7. Datsenko & Wanner (2000)[40] strategy

The Datsenko & Wanner (2000)[40] strategy for inactivation of the *nth* gene requires three transformation steps to introduce: 1) the pKD46 plasmid carrying the genes of recombineering proteins that will mediate integration of 2) the antibiotic resistance cassette flanked by FRT (flippase recognition target) sites produced by PCR amplification (from pKD13 plasmid) that will integrate the genome and 3) the pCP20 plasmid encoding the flippase gene under control of a temperature inducible promotor (thermosensitive λ repressor) (Figure 6). In previous works, all transformation steps were successful but flippase-mediated removal of the antibiotic resistance gene was not conclusive.

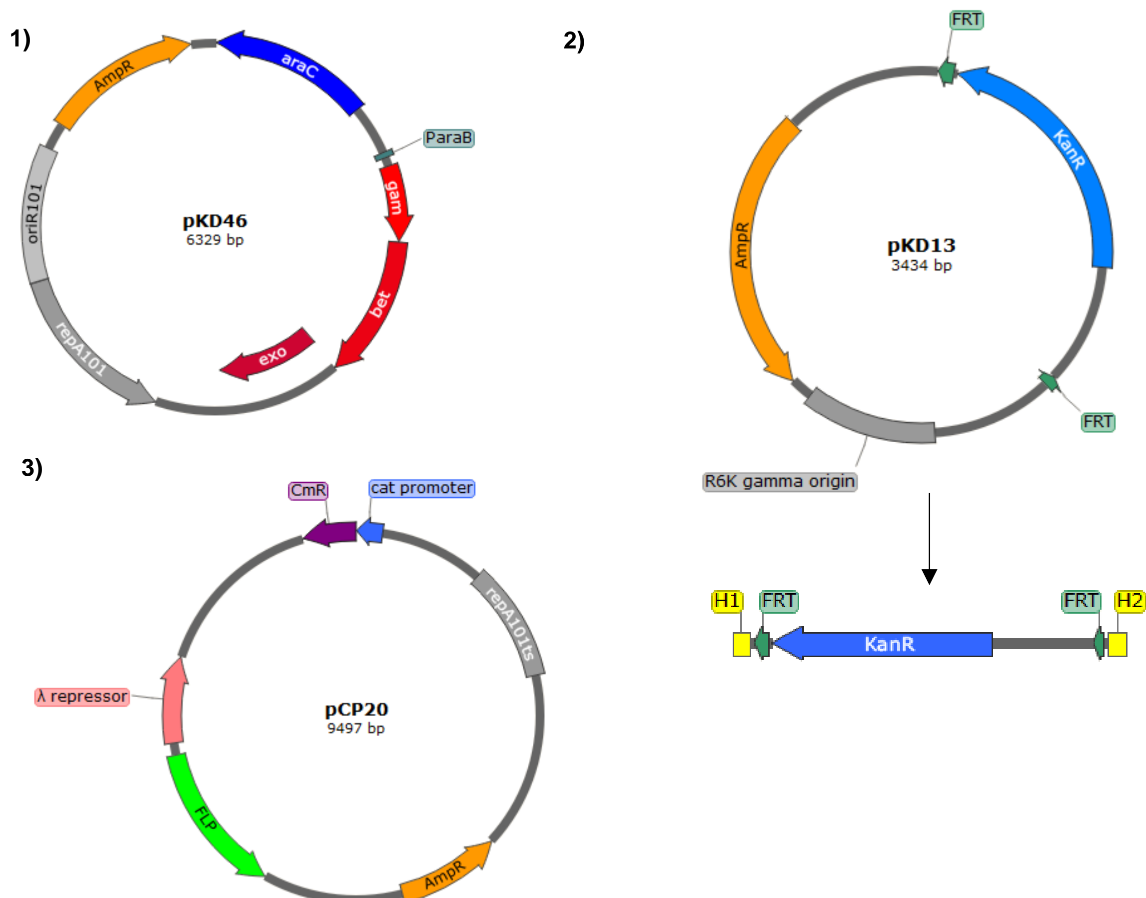


Figure 6. Map of the Datsenko & Wanner (2000)[40] plasmids: **1)** pKD46 plasmid with λ-Red recombineering genes (*gam, bet, exo*); **2)** pKD13 plasmid and amplified Kanamycin cassette (KanR) with FRT sites and homologous arms (H1/H2); **3)** pCP20 plasmid with FLP under control of thermosensitive λ repressor.

3.7.1. Confirmation of previous knockout experiments

To confirm knockout of the *nth* gene started in Duarte (2018)[62], first the presence of pKD46 plasmid in *L. lactis* LMG19460 was confirmed (by amplification of the *bla* gene present in the plasmid). The cells were grown in 5 mL GM-17 supplemented with 1000 µg/mL of neomycin at 30°C, 100 rpm, overnight. After recollection of the cells by centrifugation, pDNA purification was performed with Nucleospin Plasmid Kit (Macherey-Nagel). A PCR reaction was done with NovaTaq Hot Start Master Mix Kit (Novagen) with 10 ng of the purified pKD46 plasmid, 1x NovaTaq Hot Start Master Mix, 0.1 µM of each primer (Amp_pKD46_F/R, in Table 7) completed with PCR-grade water to a final volume of 25 µL. The

used cycling conditions were: 9 min at 95ºC, followed by 30 cycles of 30 s at 94ºC, 30 s at 60ºC and 1 min at 72ºC, and a final extension step of 10 min at 72ºC. The product of the PCR reaction was then run in an 1% agarose gel.

Table 7. Primers used in PCR amplification in the Datsenko and Wanner[40] strategy. Cassette homology arms carried by KO_nth_F/R primers in **bold**.

Primer	Sequence (5' – 3')	Product size
Amp_pKD46_F	GCGATCTGTCTATTTCGTTC	614 bp
Amp_pKD46_R	GTTCTGCTATGTGGCGCGGT	
Conf_nth_F	GTCCTCAATCGTAAGGTATC	1,007 bp (wild-type *nth* gene) 1,604 bp (cassette insertion)
Conf_nth_R	CTTTAACCACTTCTCCCGCTACC	
KO_nth_F	**agagaaagaaaccacaagaagatttttatattcctttggatggaccatgg aatagttaat**GTGTAGGCTGGAGCTGCTTC	1,414 bp
KO_nth_R	**ggtctgagccaatatcagcaagtcttgctccattatcaacataattagcta ctgctttca**TCCGTCGACCTGCAGTT	

To confirm if the tested cells had the knockout of the *nth* gene they were plated in solid regeneration medium[54] supplemented with 1,000 µg/mL of neomycin. Resulting colonies were tested via colony PCR (as described in section 3.4.) with the KOD Hot Start DNA Polymerase kit (Novagen), using the primers conf_nth_F/R. Cycling conditions were as follows: cell lysis and initial denaturation for 10 min at 95°C, followed by 30 cycles of 1 min at 95°C, 1 min at 60°C and 3 min at 70°C. The colonies that showed dubious results were plated and tested again as previously described in this section by colony PCR, but with different annealing temperatures (45°C, 50°C, 56.7°C and 63.1°C) to optimize the PCR conditions. Simultaneously their gDNA was extracted using with Wizard Genomic DNA Purification Kit (Promega). The identity of the cells was tested by colony PCR targeting the *hisG* gene.

3.7.2. Testing maximum temperature growth for induction of FLP expression

Growth at different temperatures was tested for following induction of the FLP gene in Datsenko & Wanner (2000) protocol[40]. *L. lactis* LMG19460 master cell bank was divided into equal volumes and plated in solid regeneration medium[54] and incubated at: 37°C, 39°C, 40°C, 41°C, 42°C and 43°C, until growth was observed.

3.7.3. Knock-out of the *nth* gene in *L. lactis* LMG19460

3.7.3.1. pKD46 plasmid transformation

The pKD46 plasmid, containing the genes to the λ-Red recombineering proteins, was transformed into electrocompetent *L. lactis* LMG19460 cells, using the protocol described in section 3.3.3.1. Several variables were tested to obtain the transformants: the cells were submitted to 3 or 4 electric pulses,

transformation was done with 100 ng or 500 ng of pDNA produced in *E. coli* DH5α or GM2163 strains, with or without 30 min incubation at room temperature with tetradecyltrimethylammonium bromide (TTAB) 30 mM. Some tests were also done using aliquots of electrocompetent cells prepared by a laboratory colleague. The cells were plated in solid regeneration medium supplemented with 1.5 or 2 µg/mL of ampicillin and the resulting cells were grown overnight in GM-17 with antibiotic. Cell banks were made and pDNA was purified using Nucleospin Plasmid Kit (Macherey-Nagel). Confirmation of purified plasmid identity was done by PCR amplification with the Amp_pKD46_F/R primers (in Table 7), with reaction mixture and cycling conditions as described in section 3.7.1. Colony PCR was also done using 10 µL of each cell bank and the same conditions.

3.7.3.2. Kan-cassette transformation

The kanamycin resistance cassette containing flanking FRT sites and homology arms to the *nth* gene was amplified from pKD13 plasmid, using the primers KO_nth_F/R, in Table 7. The PCR reaction was set using the KOD Hot Start DNA Polymerase kit (Novagen) with 10 ng of pKD13 plasmid, 0.02 U/µL of KOD DNA Polymerase, 2.3 mM of MgSO4, 1x buffer, 0.2 mM of dNTPs, 0.3 µM of each primer and completed with PCR-grade water to a final volume of 25 µL. The cycling conditions were: initial denaturation for 3 min at 95°C, followed by 40 cycles of 1 min at 95°C, 1 min at 55°C and 1.5 min at 70°C. The PCR product was partially (5 µL) run in a 1% agarose gel electrophoresis and, when correct, the remaining sample was column-purified with NZYGelPure – PCR clean-up (Nzytech).

Colonies containing the pKD46, were turned electrocompetent as described in section 3.3.3.1. but with antibiotic selection in every step, using 1 µg/mL of ampicillin, and the addition of L-arabinose for induction of the λ-Red recombineering proteins. Transformation of the PCR-amplified kan-cassette was done using the electroporation protocol already described, with 500 ng of DNA previously incubated with TTAB 30 mM for 30 min at room temperature and 3 or 5x electric pulses. Alternatively, the overnight recuperation step was done in 5 mL of M-17 medium, MRS medium, LB broth or Elliker liquid medium, supplemented with 0.5% of L-arabinose, 0.5% L-arabinose + 0.1% glucose or 0.5% L-arabinose + 0.5% glucose, and 1000 µg/mL of neomycin and 0.5 µg/mL of ampicillin. After an overnight incubation at 30°C without agitation in media supplemented with L-arabinose for induction of the recombineering proteins, the recovered cells were plated in regeneration medium supplemented with 2000 µg/mL of neomycin and 1 µg/mL of ampicillin.

The resulting colonies were tested by colony PCR amplification of the target region in the genome. A PCR reaction was set using a volume corresponding to 10^4 cells, 1x NovaTaq PCR Master Mix (Novagen), 5 µM of each primer (Conf_nth_F/R in Table 7) and 2 mM of $MgCl_2$, completed with PCR-grade water to a final volume of 25 µL.The cycling conditions were: 10 min at 95°C, followed by 40 cycles of 1 min at 94°C, 1 min at 60°C and 2 min at 72°C, with a final extension step of 5 min at 72ºC. The results were run in a 1% agarose gel electrophoresis. Further tests were done using extracted gDNA as template for PCR amplification in the conditions described above (without the initial step for cell lysis).

4. Results and discussion

4.1. Preliminary condition testing

4.1.1. Antibiotic concentration optimization

Both genetic engineering strategies approached in this work were optimized for use in Gram-negative bacteria, thus making it necessary to evaluate the ideal conditions for application in *L. lactis* LMG19460, a Gram-positive bacterium. In the next sections are shown some preliminary tests made to evaluate the optimized conditions in which some protocol steps had to be implemented (i.e. antibiotic concentration for selection of transformants, growth conditions for controlled gene expression).

For selection of the transformed cells it was necessary to optimize the antibiotic concentrations that allow selection for the different antibiotic resistance genes that are encoded in the plasmids, at which wild-type cells do not grow. Due to high antibiotic resistance in Gram-positive bacteria, some antibiotics had to be used in a sub-optimal concentration for selection or substituted by similar substances. Neomycin was chosen for use in the selection of kanamycin resistance since it is also an aminoglycoside antibiotic that inhibits translation of mRNA into proteins, with effective induction of the kanamycin resistance gene[69,70].

Antibiotic concentrations that allowed selection were determined again, according to the previously described method. The concentrations for testing antibiotic resistance were chosen according to the literature, previous work and the results obtained by a laboratory colleague (S. Sancho, data not published). The different concentrations for each antibiotic were tested resulting in the choosing of the following concentrations for selection with each antibiotic in the standard procedures: 1.5 µg/mL for chloramphenicol, 1,000 µg/mL for spectinomycin, 1.5 µg/mL for ampicillin and 2,000 µg/mL for neomycin. In these tests, concentrations equal to or above the selected for each antibiotic showed no colonies, whereas, concentrations bellow the selected showed a number of colonies in the same order of magnitude as the control (with no antibiotic supplementation). For spectinomycin, cells still showed growth at the selected concentration and could lead to false positives but using higher concentrations was not easy as solubility of this antibiotic is low. Later transformation tests made with pDNA carrying the spectinomycin resistance gene did not allow to conclude if this concentration was enough for selection of transformants. For future works, a more cost-effective option would be to change this selection marker in the needed plasmids.

Adjustments were made throughout the different steps in the knock-out strategies due to the sensitivity of the cells to certain procedures. In the process, described in section 3.3.3, to obtain electrocompetent cells which already have a plasmid inside or an exogenous antibiotic resistance gene (and, thus, needing selective pressure) the sensitivity of the cells (caused by the procedure)[54] only allowed growth at lower concentrations than the ones previously defined.

4.1.2. Cell viability in the presence of anhydrotetracycline (aTc)

In Reisch & Prather (2015)[49], expression of the *Cas9* gene and the sgRNA targeting the desired gene is under control of the P_{TET} inducible promoter. Tests to measure growth limitations in the presence of aTc were done to assess if the *L. lactis* strain was able to grow at the reported concentration for induction. The cells grew in the presence of the antibiotic derivative, showing no loss of cell viability as the concentrations were increased (Table 8). Although cells can grow in the reported concentration needed for induction (100 µg/mL) evaluation of the effect on expression from the tet promoter was not tested.

Table 8. Number of colonies observed in solid regeneration medium supplemented with aTc in 0, 50, 100 and 200 µg/mL

aTc concentration (µg/mL)	Number of colonies
0 (control)	76
50	83
100	114
200	135

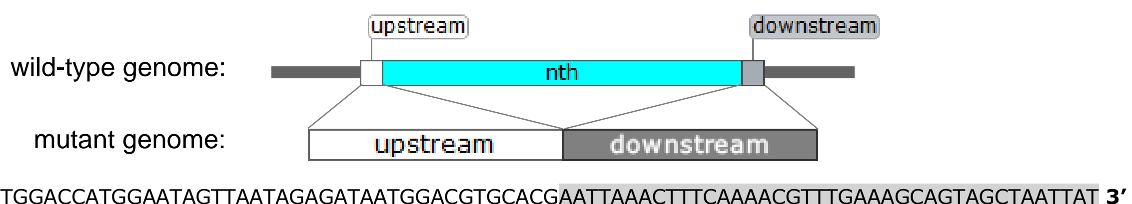
4.1.3. Maximum temperature growth

A preliminary optimization step for the Datsenko & Wanner (2000) strategy, regarding growth of LMG19460 at different temperatures was needed. The strategy requires that the FLP recombinase expression is induced by a temperature of 43°C. In Duarte (2017)[62] results showed that *L. lactis* has difficulty in growing at this temperature. Tests made with *L. lactis* LMG19460 in solid medium incubated at different temperatures (see section 3.7.2.) showed that it was able to grow at a maximum temperature of 41°C (from 30°C to 41°C, plates showed more than 300 colonies, while for temperatures above, 42°C and 43°C, no colonies appeared). This temperature is enough for later curing of the plasmid but it is still necessary to confirm that it is enough for induction of the temperature-sensitive promotor controlling FLP expression. Further tests might be needed to evaluate if growing the cells at pleasanter temperatures and then change the conditions to 43°C would also allow for induction but maintaining cell viability.

4.2. Reisch & Prather (2015)[49] strategy

4.2.1. *In silico* design of the DNA template for DSB repair following the Reisch & Prather (2017)[50] protocol

The oligo donor (ssDNA) for integration in the genome after DSB created by the sgRNA-Cas9 complex targeting the *nth* gene was designed according to the Reisch & Prather (2017)[50] protocol. For a scarless deletion of the gene, the oligo is composed of a sequence of 80 bp, 40 bp were selected upstream of the *nth* gene and the other 40 bp downstream (sequence in Figure 7). This sequence was tested *in silico* (online software mfold (http://unafold.rna.albany.edu/?q=mfold/DNA-Folding-Form)[64]) for secondary structures showing $\Delta G < - 12.5$ kcal/mol, which makes it suitable for the desired effect and there was no need of shifting the sequence.



5′ TGGACCATGGAATAGTTAATAGAGATAATGGACGTGCACGAATTAAACTTTCAAAACGTTTGAAAGCAGTAGCTAATTAT **3′**

Figure 7. Overview of the *in silico* design of the ssDNA donor (oligo), for DSB repair by integration in the genome. The final mutant cells are expected to show no scar after deletion of *nth* because the integrated oligo sequence (bottom) is equal to the flanking sequences of *nth* in the wild-type genome.

4.2.2. Cloning of pAMβ1 origin of replication in pCas9cr4 plasmid

In the Reisch & Prather (2015)[49] strategy, the first plasmid that needs to be transformed into the cells carries the *Cas9* gene under the control of the P_{TET} promoter. The pCas9cr4 plasmid has a Gram-negative specific origin of replication (p15A) that lacks a replication protein and thus, does not allow replication in hosts without the appropriate mechanisms for replication. Other plasmids in this strategy carry the specific proteins for replication so the lactococcal strain should be able to replicate them. Since it is yet unknown if the lactococcal strain possesses the necessary protein for replication of pCas9cr4 from the p15A origin of replication, the *L. lactis* LMG19460 cells were transformed with the plasmid. No results were obtained after trying to purify pDNA from transformants or by colony PCR. Although this plasmid has a low copy number, its absence suggests that the *Lactococcus* strain cannot allow replication. Cloning of a broad host range origin of replication from Gram-positive bacteria, such as pAMβ1, should turn the pCas9cr4 plasmid into a shuttle vector able to replicate in *L. lactis* LMG19460 strain[71].

To allow for future controlled experiments if the need of two different selection markers arises, the resistance to erythromycin gene was also chosen to be added to pCas9cr4 plasmid. The *L. lactis* strain is sensitive to low concentrations of this antibiotic and this result is common to other *Lactococcus* and *Lactobacillus* species[60]. The cloning of both a wide-host range origin or replication (pAMβ1) and an antibiotic resistance marker that allows easy selection in LAB, would be an advantage for application of this strategy in a variety of LAB hosts. For this, two different cloning approaches were attempted:

traditional cloning using restriction enzymes and the T4 ligase and Gibson Assembly. Although traditional cloning allows for easy directional cloning, Gibson Assembly allows efficient cloning of long fragments such as the desired for this construction[67]. Both approaches were tested for cloning a fragment with only pAMβ1 origin of replication and, in parallel, a fragment with both the origin of replication + *erm* into the *Cas9* carrying plasmid (overview of the cloning in Figure 8).

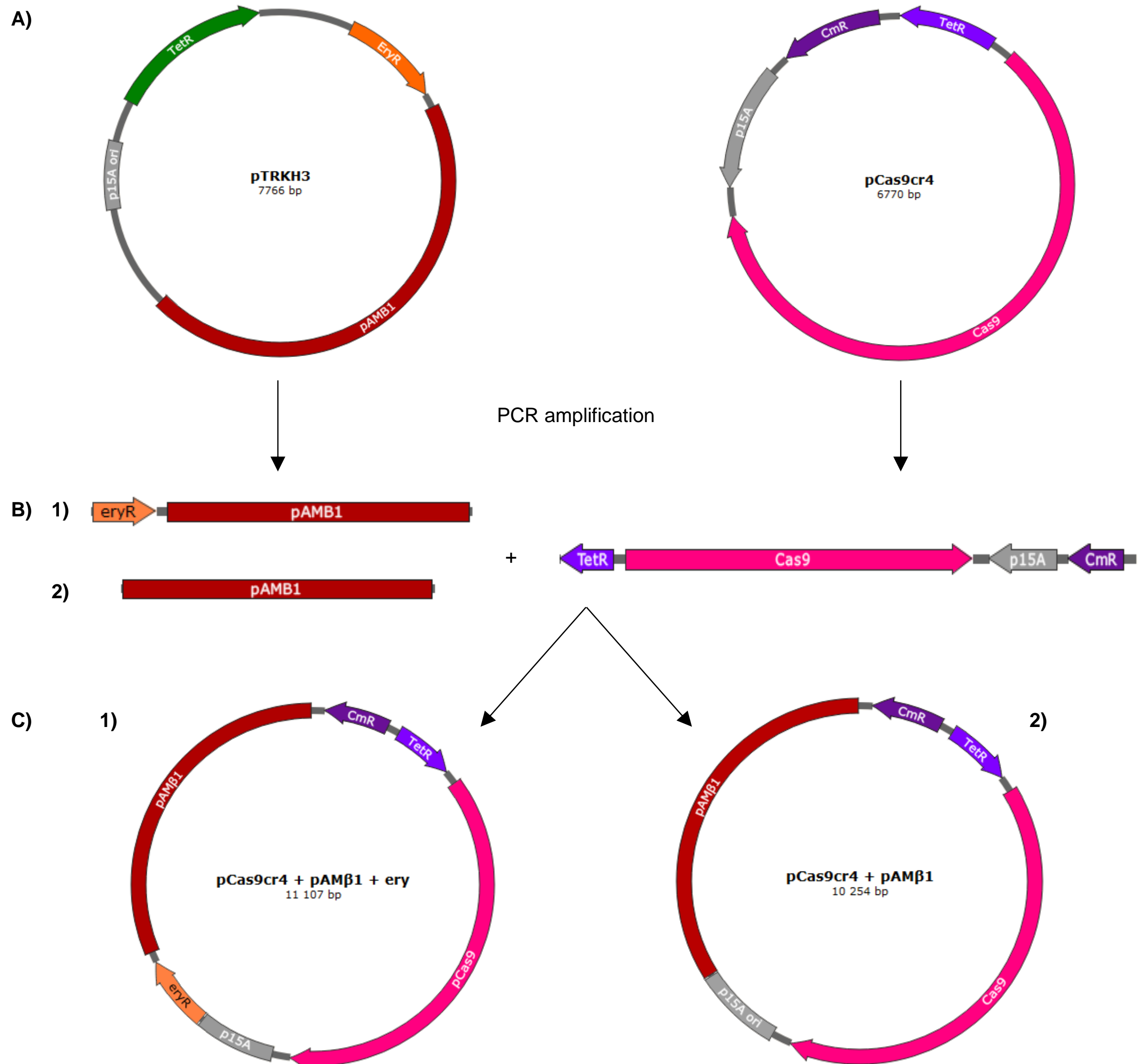


Figure 8. Overview of the desired cloning for turning the pCas9cr4 plasmid into a shuttle vector. A) pTRKH3 plasmid from which pAMβ1 origin and *erm* gene (eryR) were obtained (left) and original pCas9cr4 plasmid (right); B) on the left we can see: 1) the amplified pAMβ1 origin + *erm* gene fragment and 2) the amplified pAMβ1 origin fragment, and, on the right, the linearized pCas9cr4 vector; C) final desired constructions of the pCas9cr4 with 1) the pAMβ1 origin + *erm* gene, and 2) the pAMβ1 origin.

The cloning with both approaches was repeated more than once, varying the amount of vector and insert:vector molar ratios. An attempt at using electrocompetent *E. coli* DH5α instead of chemically competent cells was also tried, but with no results or notable differences, so, further experiments were done using the heat shock protocol for transformation.

Initial experiments using both enzyme restriction and Gibson assembly showed negative or at least dubious results with unexpected digestion patterns of the constructed plasmids. This could be caused by low concentration of the fragments and/or enzymatic inhibition and fragment modification due to extraction of the fragments from the agarose gel that could lead to defective ligation[72]. Further attempts were done without extraction from the agarose gel: so when the PCR products did not show unspecific bands, they were column purified.

The linear pCas9cr4 plasmid and the fragments pAMβ1 and pAMβ1 + *erm* gene, were successfully amplified and showed the desired length (Figure 9), meaning, the designed primers were effective for amplification. These fragments were amplified with primers that allow both cloning with restriction enzymes and Gibson assembly. For cloning with restriction enzymes, the fragments were digested and observed in an agarose gel, after confirming their size, the fragments were column purified and then used for molecular cloning.

The colonies of *E. coli* DH5α transformed with the cloning reactions, for both cloning with restriction enzymes and Gibson assembly, were inoculated in LB broth with the respective antibiotic (chloramphenicol for the construction with only pAMβ1 origin as insert, and erythromycin for the construction with pAMβ1 + *erm* gene insert). When there was growth, the pDNA from these colonies was purified and then double digested with SgsI and BsrGI restriction enzymes to confirm the presence

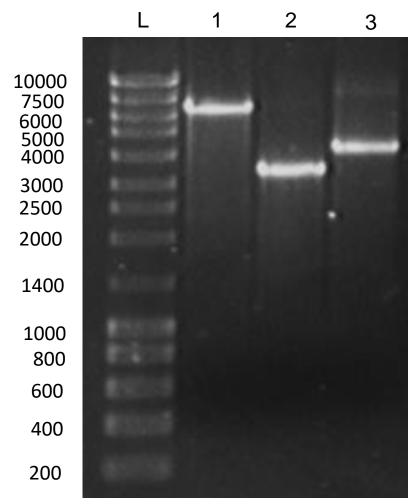


Figure 9. PCR amplification of cloning fragments for pCas9cr4 plasmid optimization. Lanes: Nzyladder III (Nzytech); 1) amplified linear pCas9cr4 plasmid (6,770 bp); 2) amplified pAMβ1 origin of replication fragment (3,518 bp); 3) amplified pAMβ1 + *erm* gene fragment (4,377 bp).

of the insert. Gibson assembly resulted in a construction with the correct digestion pattern with pAMβ1 fragment (when attempted with 3 h reaction) and with pAMβ1 + *erm* fragment (reaction times of both 1

h and 3 h) (Figure 10A). Cloning using restriction enzymes showed positive results with 3 h ligation reaction with 1:1 and 1:3 vector:pAMβ1 + *erm* fragment (Figure 10B).

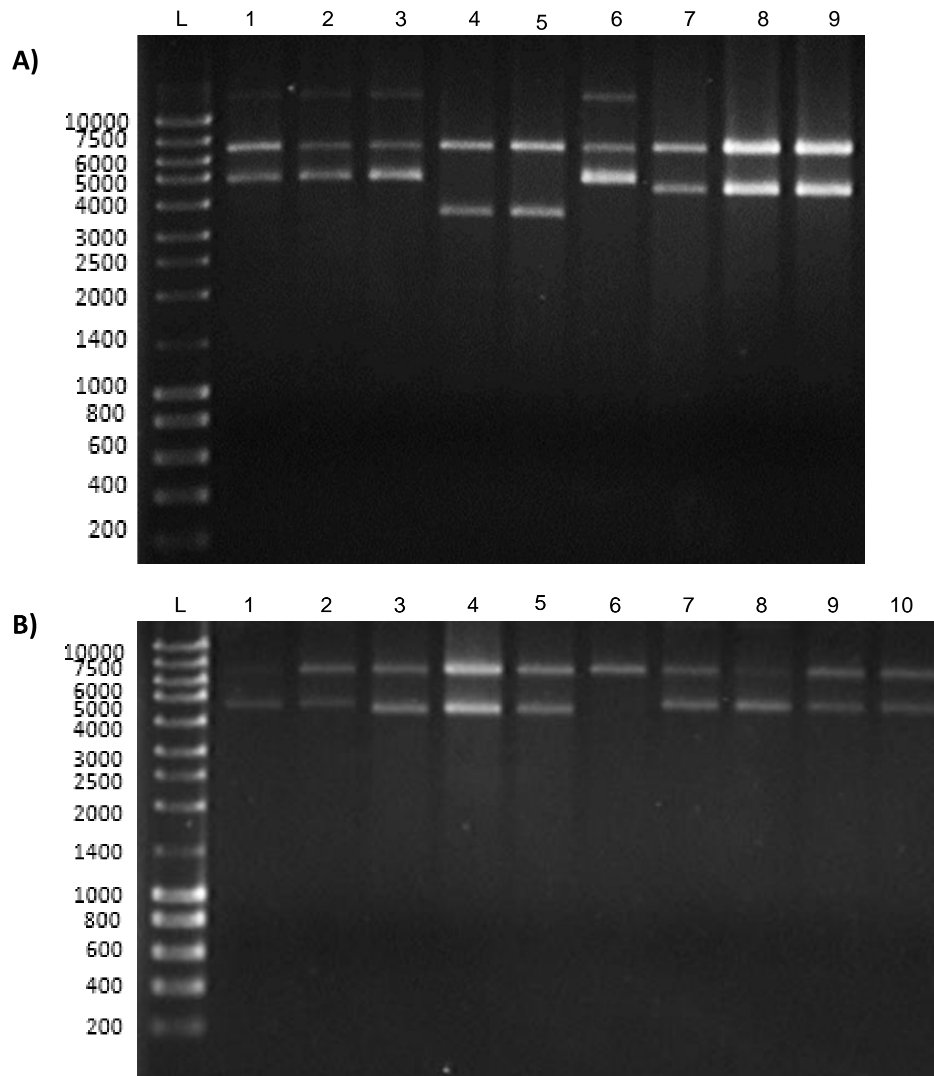


Figure 10. Double digestion with SgsI and BsrGI of pDNA obtained from the colonies resulting from the cloning process. **A)** from Gibson assembly, lanes: Nzyladder III (Nzytech), 1-3) 1 h reaction with pAMβ1 fragment, 4--6) 3 h reaction with pAMβ1 fragment, 7) 1 h reaction with pAMβ1 + *erm* fragment, 8-9) 3 h reaction with pAMβ1 + *eryR*; **B)** from cloning with restriction enzymes, lanes: Nzyladder III (Nzytech), 1-2) 3 h ligation reaction with 1:1 vector: pAMβ1 fragment, 3) 3 h ligation reaction with 1:1 vector: pAMβ1 + *erm* fragment, 4-5) 3 h ligation reaction with 1:3 vector: pAMβ1 + *erm* fragment, 6-8) overnight at 4°C ligation reaction with 1:1 vector: pAMβ1 fragment, 9-10) overnight at 4°C ligation reaction with 1:3 vector: pAMβ1 fragment. Expected band sizes were 6,770 bp + 3,518 bp/4,377 bp for pCas9cr4+pAMβ1 / pAMβ1 + *erm* plasmids.

The higher yield of positive results when cloning of pAMβ1 + *erm* fragment was observed in both cloning with restriction enzymes and Gibson assembly. Possibly due to the fact that selection of colonies was done with erythromycin, which means, theoretically, only colonies with the fragment that contains the *erm* gene should grow. However, cloning of only the origin of replication, which was selected using the original selection marker in the plasmid, chloramphenicol lead to a higher frequency of false positive colonies.

Since the positive results showed the same pattern, only one of each construction (with pAMβ1 fragment or pAMβ1 + *erm* fragment) was sent for sequencing. Both showed mutations in the pAMβ1 sequence: 2 mismatches and 1 gap in pAMβ1 fragment and pAMβ1 + *erm* fragment (*erm* had no mutations) (Figure 11). Sequencing of other samples, from both the traditional cloning or Gibson assembly, revealed that the mutations were present in the same region. The cloning process was repeated only for the pAMβ1 + *erm* fragment, using new amplified fragment and vector, and the results were similar. The positive clones showed mutations in the pAMβ1 sequence, different from the previously obtained. These mutations must have occurred during PCR amplification, even when a high fidelity polymerase was used. To overcome this, the amplification of smaller sized fragments might allow a higher fidelity, however, it would slow the cloning with these approaches since it would need sequential cloning to get the desired final plasmid.

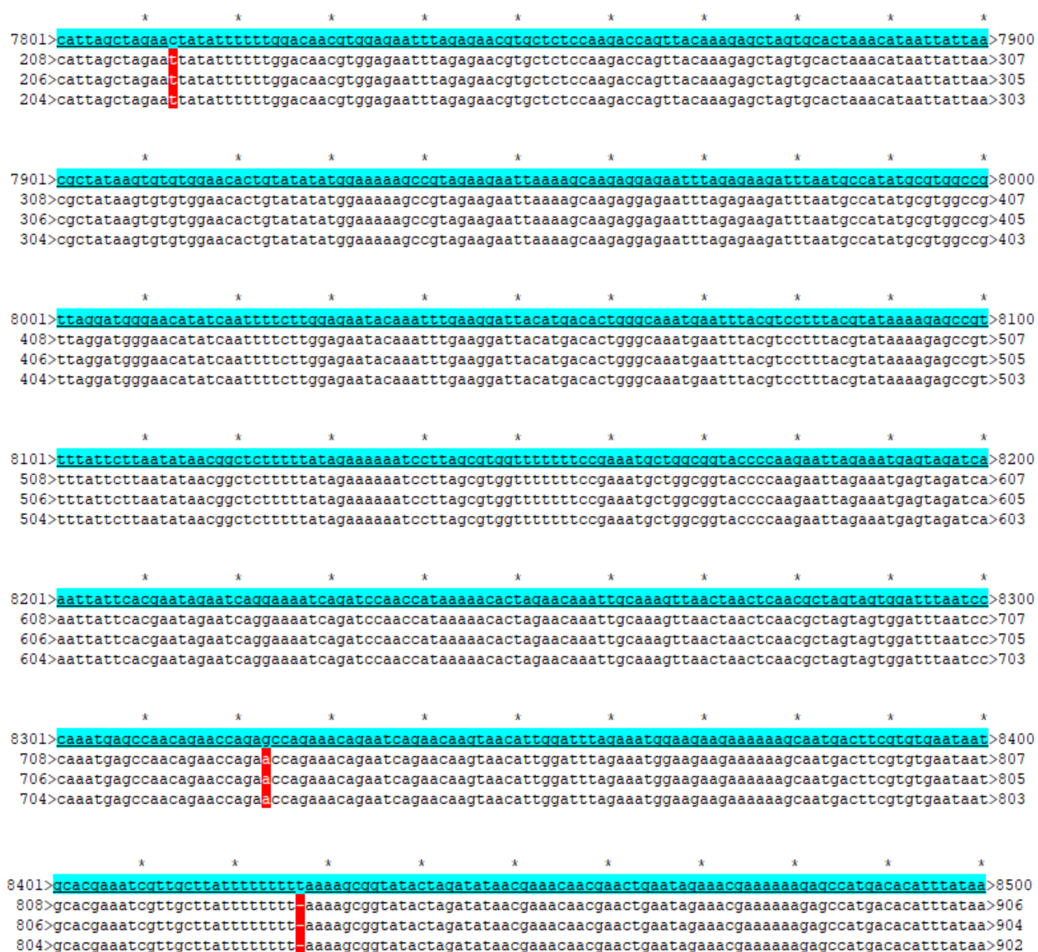


Figure 11. Alignment of the pAMβ1 origin of replication sequence (in blue, retrieved from pTRKH3 plasmid) to the results of sequencing targeting the pAMβ1 origin in the new construction of pCas9cr4 plasmid (in white) obtained by: first line) Gibson assembly with pAMβ1 fragment, second line) Gibson assembly with pAMβ1 + *erm* fragment, third line) cloning by restriction enzymes with pAMβ1 + *erm* fragment. There are 2 mismatches and 1 gap (-). Alignment was done using APE software.

One of the obtained constructions of pCa9cr4 plasmid with pAMβ1 + *erm* fragment was transformed into *L. lactis* LMG19460. Only one colony grew after inoculation in liquid medium, from which its pDNA

was purified and double digested with SgsI and BsrGI. Upon observation in an agarose gel, the plasmid showed an unexpected digestion pattern, different than the one from the construction isolated from *E. coli* DH5α. The plasmids were linearized with BamHI, BglII and Alw44I to evaluate the total length of the pDNA recovered from *L. lactis* LMG19460. The plasmid recovered from *E. coli* DH5α showed the expected size (11,147 bp and can be seen above the 10,000 bp band in Figure 12). The plasmid recovered from *L. lactis* LMG19460, however, showed a different restriction pattern when digested with the same enzymes.

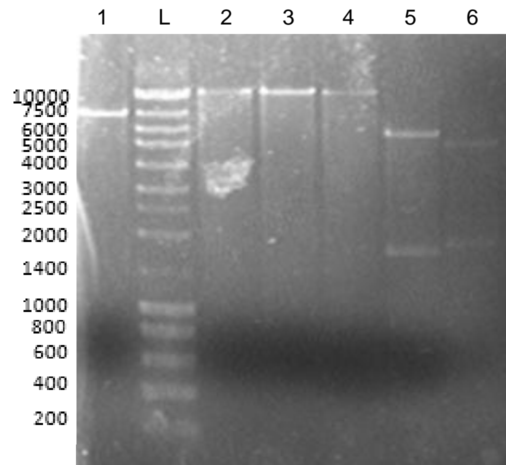


Figure 12. Digestion of pCas9cr4_pAMβ1_eryR plasmid, recovered from *E. coli* DH5α or *L. lactis* LMG19460, with different restriction enzymes. Lanes: 1) pDNA from *L. lactis* LMG19460, digestion with BamHI; Nzyladder III (Nzytech); 2) pDNA from *E. coli* DH5α, digestion with BamHI; 3) pDNA from *E. coli* DH5α, digestion with BglII; 4) pDNA from *E. coli* DH5α, digestion with Alw44I; 5) pDNA from *L. lactis* LMG19460, digestion with BglII; pDNA from *L. lactis* LMG19460, digestion with Alw44I.

Although the lactococcal strain was transformed with the plasmid recovered from *E. coli* and digested with the same enzymes, the digestion pattern suggests a loss of around 3,500 to 4,000 bp. Since *L. lactis* LMG19460 is a plasmid-free strain, possessing no endogenous pDNA, results propose that the observed plasmid corresponds to the one originally obtained from molecular cloning in *E. coli* DH5α and transformed into *L. lactis* LMG19460, that suffered some rearrangements throughout the process. The plasmid obtained from *L. lactis* was sent for sequencing and the presence of the pAMβ1 + *erm* fragment was confirmed (Figure 13 and 14, respectively), but maintaining the mutations observed in the plasmid recovered from *E. coli* (in Figure 11). All sequencing steps were done with the primers in Table 6.

A)

```
7601>cagcttccaaggagctaaagaggtccctagcgctcttatcatggggaagctcggatcatatgcaagacaaaataaactcgcaacagcacttggagaaatg>7700
   9>accgcttccaggagctaaagaggtccctagcgctcttatcatggggaagctcggatcatatgcaagacaaaataaactcgcaacagcacttggagaaatg>108

7701>ggacgaatcgagaaaaccctctttacgctggattacatatctaataaagccgtaaggagacgggttcaaaaaggtttaaataaaggagaagcaatcaatg>7800
 109>ggacgaatcgagaaaaccctctttacgctggattacatatctaataaagccgtaaggagacgggttcaaaaaggtttaaataaaggagaagcaatcaatg>208
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B)

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10901>ttgaataggtcatttaagttgagcatattagaggaggaaaatcttggagaaatatttgaagaacccgattacatggattggattagttcttgtggttacg>11000
  234<ttgaataggtcatttaagttgagcatattagaggaggaaaatcttggagaaatatttgaagaacccgattacatggattggattagttcttgtggttacg<135

11001>tggtttttaactaaaagtagtgaatttttgatttttggtgtgtgtgtcttgttgttagtatttgctagtcaaagtgattaaatagaattccggatgagc->11099
  134<tggtttttaactaaaagtagtgaatttttgatttttggtgtgtgtgtcttgttgttagtatttgctagtcaaagtgattaaatagaattccggatgagca<35
```

Figure 13. Alignment of the pAMβ1 sequence (in blue, retrieved from pTRKH3 plasmid) to the results of sequencing targeting pAMβ1 ori in the new construction of pCas9cr4 plasmid retrieved from *L. lactis* LMG19460 (in white): **A)** beginning of the pAMβ1 ori sequence (sequenced with primer pAMβ1_conf_beg), **B)** end of the pAMβ1 ori sequence (sequenced with primer pAMβ1_conf_end). This figure shows only the beginning and ending of the sequence, it does not show the mismatches or gaps present in the sequence. Alignment was done using APE software.

```
   1>~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ctggaaatagttgttactagtgtacaccggattacatgaacaaaaatataaaa>53
6701>atttcagtgcaatttatctcttcaaatgtagcacctgaagtcagccccatacgatataagttgttactagtgtacagattacatgaacaaaaatataaaa>6800

  54>tattctcaaaactttttaacgagtgaaaaagtactcaaccaaataataaaacaattgaatttaaaagaaaccgataccgtttacgaaattggaacaggta>153
6801>tattctcaaaactttttaacgagtgaaaaagtactcaaccaaataataaaacaattgaatttaaaagaaaccgataccgtttacgaaattggaacaggta>6900

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6901>aagggcatttaacgacgaaactggctaaaataagtaaacaggtaacgtctattgaattagacagtcatctattcaacttatcgtcagaaaaattaaaact>7000

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7001>gaatactcgtgtcactttaattcaccaagatattctacagtttcaattccctaacaaacagaggtataaaattgttgggagtattccttaccatttaagc>7100

 354>acacaaattattaaaaaagtggtttttgaaagccatgcgtctgacatctatctgattgttgaagaaggattctacaagcgtaccttggatattcaccgaa>453
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 454>cactagggttgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaat>553
7201>cactagggttgctcttgcacactcaagtctcgattcagcaattgcttaagctgccagcggaatgctttcatcctaaaccaaaagtaaacagtgtcttaat>7300

 554>aaaacttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtcaactgttt>653
7301>aaaacttacccgccataccacagatgttccagataaatattggaagctatatacgtactttgtttcaaaatgggtcaatcgagaatatcgtcaactgttt>7400

 654>actaaaaatcagtttcatcaagcaatgaaacacgccaaagtaaacaatttaagtaccgttacttatgagcaagtattgtctattttaatagttatctat>753
7401>actaaaaatcagtttcatcaagcaatgaaacacgccaaagtaaacaatttaagtaccgttacttatgagcaagtattgtctattttaatagttatctat>7500

 754>tatttaacgggaggaaataattctatgagtcgcttttgtaaatttggaaagttacacgttactaaagggaatgtagataaattattaggtatactactga>853
7501>tatttaacgggaggaaataattctatgagtcgcttttgtaaatttggaaagttacacgttactaaagggaatgtagataaattattaggtatactactga>7600
```

Figure 14. Alignment of the *erm* gene sequence (in blue, retrieved from pTRKH3 plasmid) to the results of sequencing targeting *erm* in the new construction of pCas9cr4 plasmid (in white) retrieved from *L. lactis* LMG19460. No mismatches or gaps were observed. Alignment was done using APE software.

It is still necessary to evaluate if the other relevant features of pCas9cr4 plasmid are present in this construction. However, the size discrepancy between the two plasmids recovered from *L. lactis* and *E. coli* (3,500-4,000 bp) happens to be similar to the size of the *Cas9* gene (4,104 bp), it is then possible

that the transformed plasmid lost this gene. Reports show that pDNA might suffer rearrangements when the host is under stress, the presence of an exogenous endonuclease, Cas9, might create enough instability[73]. In the original Reisch & Prather strategy, the *Cas9* gene is under control of an inducible promoter and can only be expressed in the presence of aTc, however, this happens in a plasmid with a weaker origin of replication than pAMβ1. If leaky expression where to occur, the Cas9 protein could be expressed and be toxic to the cell. Further tests would need to be done to confirm this hypothesis.

4.2.3. Cloning of sgRNA targeting *nth* gene in pKDsgRNA-p15 plasmid

The second step to optimize the Reisch & Prather (2015)[49] strategy was the design and cloning of the sgRNA targeting the *nth* gene into the pKDsgRNA-p15 plasmid (Figure 15A). The *in silico* design was done so that the sgRNA was complementary to a region of the *nth* gene adjacent to a PAM sequence (5' GGG 3', in this case) and no off-target activity was observed using online software Cas-OFFinder[66], so the sequence with the best result was chosen as sgRNA.

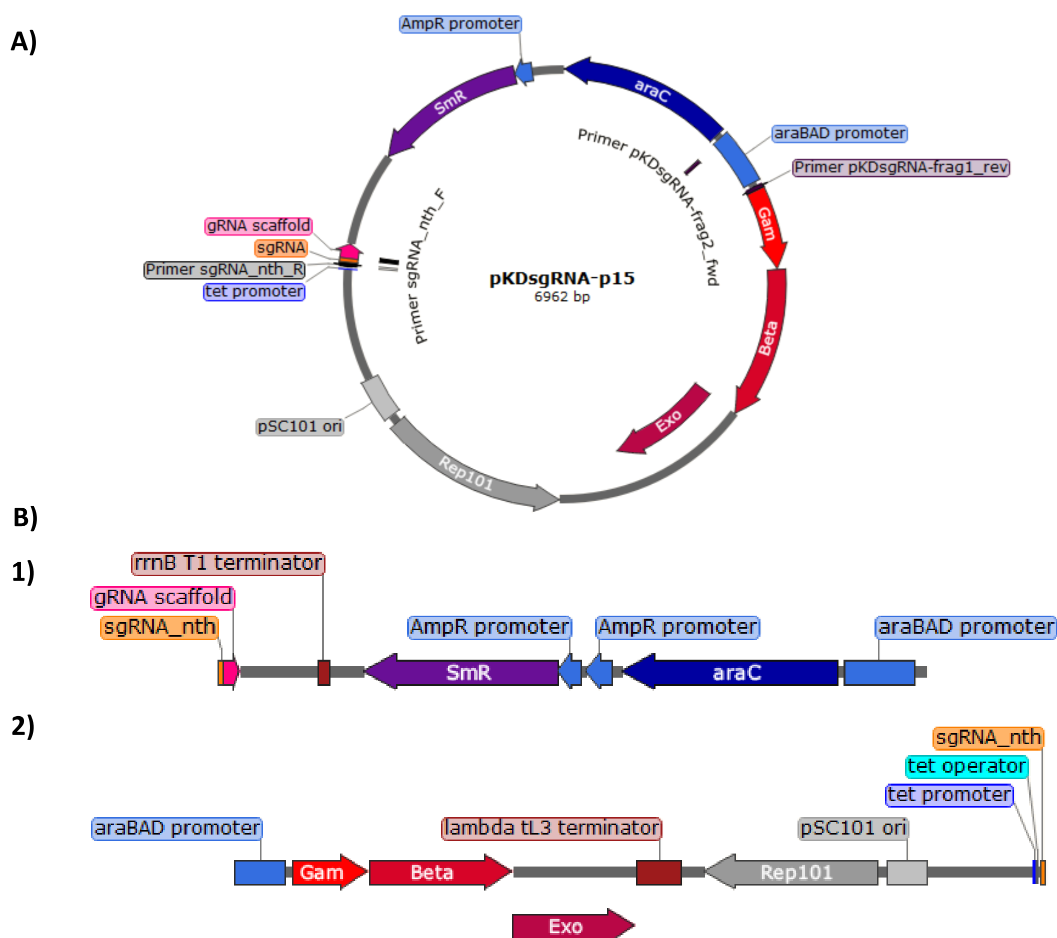

Figure 15. A) Map of pKDsgRNA-p15 plasmid. **B)** *In silico* design of the desired fragments for cloning of sgRNA targeting the *nth* gene: **1)** fragment 1 amplified with sgRNA_nth_F and pKDsgRNA-frag1rev primers, yielding a 2,868 bp fragment ; **2)** and fragment 2 amplified with sgRNA_nth_R and pKDsgRNA-frag2fwd primers giving a 4,434 bp fragment. Ligation of this fragments was done by PCR exploiting the homologous regions in the fragments (sgRNA and P_{araB} promoter) for annealing.

Cloning was done with CPEC, as described in section 3.6.3. An overview of the amplified fragments with the sgRNA (Figure 15B), shows that ligation is achieved because of the complementary extremities of the fragments (sgRNA and P_{araB} promoter).

Two fragments of the pKDsgRNA-p15 plasmid were amplified with designed primers (Table 4) that allowed the addition of the sgRNA targeting the *nth* gene. These fragments were run in a 1% agarose gel, showing bands with the expected size (Figure 16). After extraction from the gel, the fragments were cloned by PCR using their homologous regions for annealing, and the product was transformed into *E. coli* DH5α cells.

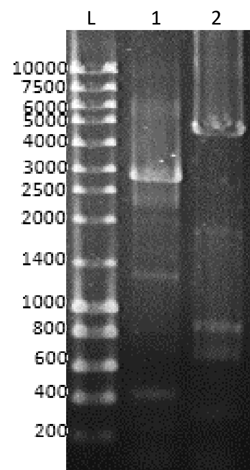

Figure 16. PCR products of amplification for cloning of sgRNA targeting *nth* gene. Lanes: Nzyladder III (Nzytech); product of amplification of pKDsgRNA-p15 with: 1) sgRNA_nth_F and pKDsgRNA-frag1rev primers (2,868 bp); 2) sgRNA_nth_R and pKDsgRNA-frag2fwd primers (4,434 bp).

After cloning the sgRNA targeting the *nth* gene in the plasmid pKDsgRNA-p15, the pDNA from the cells was purified and sent to sequencing. Four samples were sent to sequence and only one exhibited the correct sgRNA sequence. Alignment to the *L. lactis* LMG19460 genome shows that the cloned sgRNA targets correctly the *nth* gene (Figure 17).

```
Matches(|):20              GCAGAAGCCTACGGAATTCC~~~
Mismatches(#):0            GCAGAAGCCTACGGAATTCCGGG
Gaps( ):0
Unattempted(.):0
```

Figure 17. Alignment of the single-guide RNA (underlined) targeting *nth* gene from the *L. lactis* LMG19460 genome (blue), next to a PAM sequence (5' GGG 3') to the done in APE software.

The colony with the correct construction was grown in liquid medium and aliquots were stored at -80°C. This plasmid is already constructed and ready for use in the deletion of *nth* via the no-SCAR strategy[49].

However, this plasmid was not yet introduced into *L. lactis* because the strategy requires that the pCas9cr4 plasmid is present in the cells first. The pCas9cr4 needs to be functional and inside the cells, so that expression of TetR repressor inhibits the production of the Cas9 protein (controlled by the tet promoter). If the pKDsgRNA-nth plasmid is introduced first, the repressor is not present and the sgRNA

is expressed without induction. Upon introduction of pCas9cr4 in the cells, the Cas9-sgRNA is rapidly formed and starts cutting the genome at the target site without the presence of the ssDNA oligo to repair the DSB, leading to cell death. Since the plasmids and ssDNA are individually transformed into the cells, it is necessary to introduce them in a sequential order so that the system can be controlled with the tet promoter.

4.3. Datsenko & Wanner (2000)[40] strategy

4.3.1. Confirmation of *nth* gene knockout from previous work[62]

The Datsenko & Wanner strategy was already started by Duarte (2018)[62] and effective results were shown in every step, but the final removal of the resistance gene was not complete. To confirm the work until here done, the *L. lactis* LMG19460 cells were tested for the presence of the pKD46 plasmid (first plasmid needed in the gene inactivation strategy) by PCR with Ampl_pKD46_F/R primers and the extracted pDNA as template. The results were positive for the presence of the plasmid inside the cells (Figure 18), which means, the second step of the strategy (integration of the kanamycin resistance cassette) needed to be confirmed.

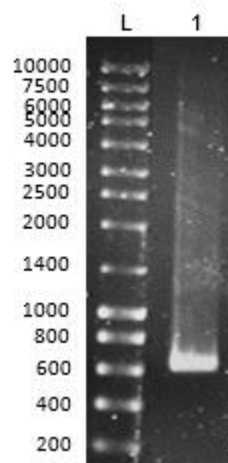

Figure 18. PCR confirmation of pKD46 plasmid presence. Lanes: Nzyladder III (Nzytech); 1) amplified fragment of the *bla* gene (614 bp) from pDNA extracted from *L. lactis* LMG19460 with Ampl_pKD46_F/R primers.

The cells containing the pKD46 plasmid, were plated in solid medium and tested for the presence of the *nth* gene versus kanamycin resistance cassette in the genome by colony PCR with the Conf_nth_F/R primers. From six tested colonies, three showed a dubious result with both the fragment amplified when *nth* gene was present (wild-type genotype, with 2,790 bp) and the kanamycin cassette (mutant genotype, with 2,133 bp). One of these colonies was tested again by colony PCR but using different annealing temperatures (as described in section 3.7.2.) for the primers to try to eliminate unspecific bands, with no results.

The colonies yielding dubious results by colony PCR were isolated and genomic DNA extraction was done. The gDNA was used as template for a PCR reaction targeting the *hisG* gene for confirmation of the identity of the cells, but with no results. For precaution, the strategy was re-made from the beginning with a new cell master bank of *L. lactis* LMG19460.

4.3.2. Knock-out of the *nth* gene in L. lactis LMG19460

4.3.2.1. pKD46 plasmid transformation

After evaluation of the cells obtained by Duarte (2018)[62] using the Datsenko & Wanner (2000)[40] strategy, it was necessary to retry the knock-out strategy of the *nth* gene. Firstly, the pKD46 plasmid was transformed into electrocompetent *L. lactis* LMG19460 cells. First attempts were unsuccessful due to the need of optimization of the used antibiotic concentration. The work of Duarte (2018)[62] selected the transformed cells with ampicillin at a 100 times higher concentration than the effective in the present work, with the cells dying at concentrations as low as 1.5 µg/mL[62]. After optimization of the antibiotic concentration, transformation was attempted as described in section 3.3.3, using the standard electroporation protocol, with still no conclusive results.

The pKD46 plasmid needs to be in a low-copy number inside the cells to decrease the probability of undesired leaky expression of the recombineering genes, that could lead to off-target recombination events. However, this also causes a low expression of the β-lactamase (*bla* gene), slowing the process of selection of positive transformants. Due to the low copy number nature of the plasmid and the high sensitivity of the cells to the antibiotic used for selection, effectiveness of the process of transformation was not easily evaluated.

Several variables where then tested, varying: amount (100 ng or 500 ng of pDNA) and host of origin of pDNA (purified from *E. coli* DH5α or GM2163 that allows for unmethylated pDNA), number of electric pulses (3x, 4x or 5x) and incubation with a detergent (with and without TTAB). These conditions were varied to try to increase the number of transformants. *E. coli* GM2163 produces unmethylated pDNA, which has been reported to increase transformation efficiency[74]. TTAB is a detergent that allows the compaction of DNA inside micelles, acting as carriers of DNA to the inside of the cell and protecting it from degradation[75]. It was observed that the transformation with 100 ng of pDNA from *E. coli* DH5α with TTAB incubation using 3x electric pulses resulted in less colonies (21) than the other sets of variables (all of them with >300 colonies resulting from transformation).

Confirmation of the presence of the plasmid was attempted by colony PCR with primers targeting the *bla* gene (ampicillin resistance) in the plasmid, with no results. A PCR reaction in the same conditions but using extracted pDNA as template yielded positive results for every set of variables tested that resulted in isolated colonies (Figure 19).

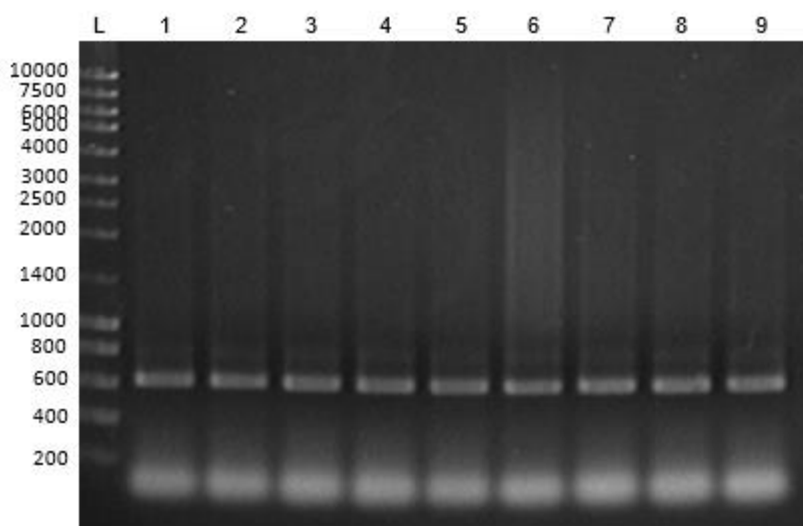

Figure 19. Lanes: Nzyladder III (Nzytech); PCR amplification with Ampl_pKD46_F/R primers targeting the *bla* gene (614 bp) of pDNA extracted from L. lactis LMG19460 after transformation in the following conditions: 1) with 30 min TTAB 30 mM incubation, 100 ng of pKD46 from E. coli DH5α. 3x pulses; 2) with 30 min TTAB 30 mM incubation, 100 ng of pKD46 from E. coli DH5α, 4x pulses; 3) 100 ng of pKD46 from E. coli DH5α 3x pulses; 4) 100 ng of pKD46 from E. coli DH5α, 4x pulses; 5-6) 500 ng of pKD46 from E. coli DH5α, 3x pulses, electrocompetent cells prepared by a laboratory colleague; 7-8) 500 ng of pKD46 from E. coli DH5α, 3x pulses; 9) 100 ng of pKD46 from E. coli GM2163, 4x pulses

4.3.2.2. Kan-cassette transformation

The positive colonies were turned electrocompetent, as described in section 3.3.3.1, the process was done in the presence of antibiotic for maintenance of the pKD46 plasmid and L-arabinose for induction of the λ-Red recombineering proteins. The conditions used for transformation were the same as described in previous works[62]. The overnight recuperation step, however, was done maintaining induction of the proteins by L-arabinose in M-17 medium, MRS medium, LB broth or Elliker liquid medium, to investigate the effect of different media composition on the use of L-arabinose for induction. The colonies obtained from the experiments using Elliker medium did not grow, the others were grown in liquid medium and tested by colony PCR targeting the genomic region of the *nth* gene, with the necessary volume of culture to obtain 10^6, 10^5 and 10^4 cells. The only result was obtained in the colony PCR using 10^4 cells, showing no integration of the kan-cassette. The colonies were also tested by PCR amplification using their extracted gDNA as template. The same gDNA was used for PCR amplification of the *hisG* gene to confirm the identity of these cells as *L. lactis* LMG19460. The results showed that the desired strain was present. However, it was present only as wild-type, showing no integration of the kan-cassette into the genome. This experiment was repeated using MRS and M-17 media supplemented with L-arabinose for the overnight recuperation step since these were the conditions that allowed for better growth and recovery of colonies. From each set of conditions, 10 to 15 colonies were inoculated into GM-17 with neomycin, the ones that presented growth, only 13 total, were recovered and tested by PCR amplification using gDNA but all had the wild-type genome (in Figure 20 we can see the results for two of these colonies, the results were the same in all 13).

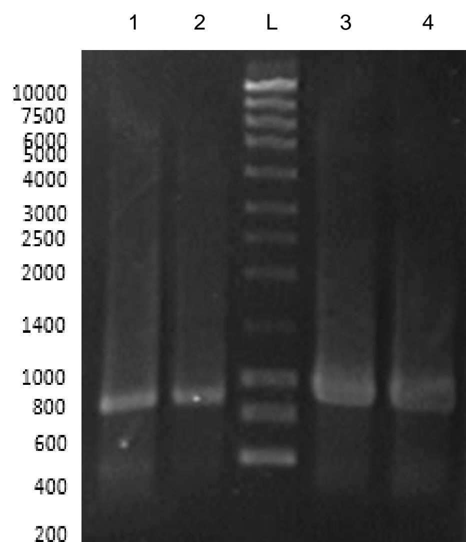

Figure 20. PCR amplification targeting the *hisG* gene and *nth* gene regions in the genome. Lanes: 1-2) amplification of the *hisG* gene from the gDNA obtained from two different colonies (933 bp); Nzyladder III (Nzytech); 3-4) amplification of the *nth* gene from the gDNA obtained from two different colonies (wild-type = 1,007 bp, with integrated kan-cassette = 1,604 bp).

The Holo & Nes (1989)[54] electrocompetence protocol, used as standard for *L. lactis* LMG19460 in the laboratory is described in 3.3.3., and uses glucose and sucrose in the process. The presence of these sugars creates catabolic repression blocking the usage of L-arabinose by the bacteria that might not allow the induction of the λ-Red proteins for integration of the kan-cassette into the genome. Alternatively, the Palomino *et al.*[58] high salt concentration protocol for electrocompetence, that does not require the addition of sugars, was tested. However, *L. lactis* LMG19460 seem to have difficulty growing in MRS medium supplemented with the antibiotic needed to maintain the pKD46 plasmid, even in lower concentrations than the assessed previously. Moreover, the presence of salt in high concentration hinders the growth, making it difficult to apply this protocol even in other media, such as M-17. Both MRS and M-17 media supplemented with 0.2% L-arabinose and 0.75 or 0.25 µg/mL of ampicillin were used to test this hypothesis with no observed growth after more than three overnights.

The use of different media supplemented with only L-arabinose for the overnight recovery step could help forcing the induction of the recombineering genes, although, most media already have other sugars (such as glucose or lactose). There are reports of other *L. lactis* strains, specially plant-associated bacteria, using L-arabinose for fermentation[76]. Wild-type cells were inoculated into MRS, M-17, LB broth and Elliker medium supplemented with L-arabinose, in which Elliker and LB did not show significant growth even after incubation for three consecutive overnights. *L. lactis* LMG19460 is apparently not able to show significant growth in minimal medium with only L-arabinose as a carbon source.

5. Overview and Conclusions

The main objective of this work was to apply genetic engineering tools to begin optimization of a LAB strain and create a safe and food-grade cell-factory for production of pDNA and recombinant proteins. LAB have a great potential as producers of high quality pharmaceutical molecules, however, most need improvement to achieve high plasmid yields and, consequently, these molecules. The *L. lactis* LMG19460 strain was chosen for knock-out of the *nth* (endonuclease) gene. The inactivation of this gene would allow the introduction of pDNA into the cells without degradation, which is the main setback for the use of this strain, as verified in Duarte (2018)[62]. The overcoming of this problem would allow easier genome editing and optimization of this strain for high yield production of pDNA of interest.

For this, two genome editing strategies designed in Gram-negative bacteria were chosen for use and optimization in *L. lactis*: the Reisch & Prather (2015)[49] no-SCAR strategy and the Datsenko & Wanner (2000)[40] recombineering strategy. None of the strategies was successfully carried out, with some optimization steps still not achieved.

The first strategy couples recombineering with a CRISPR-Cas9 system, it allows for a scarless knock-out of the gene using the Cas9-sgRNA complex to cut the target gene and the λ-Red recombineering proteins to integrate a ssDNA oligo in the genome. The ssDNA oligo was designed to have homology to the regions immediately flanking the *nth* gene, to leave no scar after integration. The sgRNA was successfully introduced into the pKDsgRNA plasmid, carrying also the *λ-Red* genes. The first plasmid to be introduced for application of this strategy, however, required modifications to allow replication and introduction into LAB. A wide-host range origin of replication (pAMβ1) and an antibiotic resistance marker to which this group shows high susceptibility (erythromycin), were cloned into the pCas9cr4 plasmid, carrying the *Cas9* gene. After introduction of the obtained construction into *L. lactis* LMG19460, the plasmid showed a loss of around 4,000 bp. This is a similar to size to the *Cas9* gene (4,104 bp), it is then possible that the transformed plasmid lost this gene, but further tests would need to be done for confirmation. If the gene was lost, it is then necessary to retry the cloning of the pAMβ1 and *erm* gene into the pCas9cr4 plasmid. To overcome mutations created in amplification of the fragments, perhaps it is best to use a strategy that requires smaller fragments. The pKDsgRNA-nth plasmid was not introduced into *L. lactis* LMG19460 because the strategy requires the first plasmid to be inside the cells first. Moreover, tests with the spectinomycin show that this strain is also highly resistant to this antibiotic, so changing of the selection marker in pKDsgRNA plasmids, might be necessary for application in this strain.

The recombineering strategy uses the λ-Red proteins to integrate an antibiotic resistance cassette flanked by FRT sites into the target region in the genome. The plasmid carrying the recombineering proteins under control of the L-arabinose inducible promoter was effectively introduced into *L. lactis* LMG19460. However, integration of the kanamycin cassette was not achieved. This could be due to the inability of the strain to use L-arabinose as the sole carbon source for growth (as confirmed in this work). The presence of other sugars for growth of the strain in liquid media and in the electrocompetence protocol, inhibits the use of L-arabinose for induction of expression. Since the λ-Red proteins are not

expressed, it is impossible to integrate the cassette and remove the *nth* gene. On the other hand, even if integration occurred, the lack of observed transformants might be due to the difficulty of selection. This strain is highly resistant to kanamycin/neomycin, making the selection arduous even in high antibiotic concentrations. Although this strategy has been applied in other LAB bacteria, for application in *L. lactis* LMG19460 it would be necessary to use a different inducible promoter (such as the xylose-inducible promoter or the NICE expression system[6,77]). Moreover, the reported applications of this strategy in LAB, have used other antibiotic resistance cassettes, to which the bacteria are more susceptible[36]. In the laboratory tests described in the present work, results show that ampicillin, chloramphenicol or erythromycin would be better for selection of transformants, since this strain is highly sensitive to these. To optimize this strategy for a wide-range application in LAB, these would still be a good choice as their effect is vastly spread in several strains in this group[60].

It is possible that the greatest setback in application of these strategies in *L. lactis* LMG19460 is the need to transform several DNA molecules sequentially. Since the strain has a high rate of exogenous DNA degradation, it is likely that the plasmids and linear DNA do not stay intact in the cells enough time to introduce the next piece of DNA in the strategy. In addition, several steps were needed to optimize transformation of each molecule (media, antibiotic concentration, temperature, etc.), making the successive steps in these approaches arduous. The use of a strategy that requires only one plasmid carrying all the necessary machinery for genome editing, would be advised in this strain. Some have already been applied to Gram-positive bacteria and LAB, using a plasmid carrying both the Cas9 endonuclease, the sgRNA and the homology arms for repair of the DSB[78]. Other reports show the application in *E. coli* of a strategy that does not require homologous DNA template for repair of DSB, hijacking the cell's own DNA repair system[79].

This work allowed a different perspective regarding the optimization and engineering of LAB strains, making it another step towards the construction of a safe GRAS bacterial alternative for the production of pharmaceutical-grade pDNA and recombinant proteins.

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