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

Rita Pires[a] | **Supervisor:** Dr. Sofia de Oliveira Dias Duarte, **Co-Supervisor:** Prof. Gabriel António Amaro Monteiro
[a]*Laboratories of Bioengineering Research Group (BERG), Institute for Bioengineering and Biosciences (IBB), Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisbon, Portugal*

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 and safe strains for these applications makes it essential to find appropriate genetic engineering tools for LAB genome editing. 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 plasmid (pKDsgrRNA-nth) was not transformed. 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. The use of a different inducible promoter and antibiotic resistance cassette may be preferred. 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.

Introduction

Lactic Acid Bacteria (LAB) have been used for centuries in food fermentation but more recently there has been a wider research interest both in academia and industry. Their genetic and biochemical properties make them ideal not only for different industrial fermentation processes but for new medical approaches and therapies. *Lactococcus lactis* strains role in the dairy industry has made it one of the most studied LAB species. New studies regarding their physiology and genetics has led to breakthrough discoveries that point LAB as being suited for several new applications: as cell-factories for pharmaceutical-grade plasmid DNA (pDNA), recombinant proteins or metabolite production, and *in situ* production of therapeutically relevant molecules, as live bacterial vectors for delivery of biopharmaceutical-grade molecules[1,2]. Although the traditional 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[3]. LAB are a safer and promising alternative. Their GRAS status, non-pathogenicity and the ability to

colonize human mucosal surfaces makes them an especially promising tool in the use of mucosal DNA vaccines[4].

In traditional applications of LAB, the use of genetically modified strains is not within regulations, so many of these bacteria are not optimized for production of heterologous plasmid DNA or proteins. For novel medical applications, however, it is imperative to engineer strains that can deliver safely and effectively the desired therapeutic molecules. For live mucosal vaccination, for example, it is necessary that the bacterial vector is able to produce chosen antigens and present them in enough concentration for induction of an immune response[4]. Thus, new modified and improved strains must be designed to meet the requirements of these strategies[5].

Many genetic engineering processes have been reported in different LAB species but the need for an optimized pharmaceutical-grade method for genome editing in *L. lactis* is still in need. For safe and effective use of these strains it is imperative to minimize health concerns, so it is necessary to find genome editing strategies that allow mutations in the genome without leaving antibiotic resistance genes inside the cell. Furthermore, an optimized wide host range genome editing strategy would provide breakthrough solutions for innovation in applications of LAB[5].

Recombineering is a technology developed in *E. coli* but several methods have been reported in LAB[5,6]. It relies on the expression of three λ-red phage proteins: Beta, Gam and Exo, that mediate recombination of a DNA template (double- (dsDNA) or single-stranded DNA (ssDNA)) homologous to a target sequence in the genome. In Datsenko & Wanner (2000)[7] the authors use this strategy to successfully disrupt several chromosomal genes in *E. coli*. The process allows replacement of a target sequence with an FRT-flanked antibiotic resistance cassette generated by PCR using primers with homology extensions to the target site, and the homologies recombine with the genome by Red-mediated recombination. After selection, this resistance marker is removed by using a plasmid expressing FLP recombinase that will act on the FRT sites flanking the resistance gene. The used plasmids can then be cured because of their temperature-sensitive origins of replication, leaving no selection markers within the cell[7]. This strategy has been reported in *L. lactis* spp. *cremoris* MG1363 for construction of a thymidylate synthase (*thyA*) deficient strain but with no removal of the antibiotic resistance gene[8]. An attempt to use this strategy for modification of a *Lactococcus lactis* spp. *lactis* strain (LMG19460) was made by Duarte (2018)[9] in which successful removal of the antibiotic cassette was not yet obtained. This was due to the inability of this strain to grow at the temperature needed for inducing expression of FLP.

In Reisch & Prather (2015)[10] the authors develop a Scarless Cas9 Assisted Recombineering (no-SCAR) system in *E. coli*, that allows easy and fast subsequent modifications, coupling recombineering with a CRISPR-Cas9 system. In contrast to the previously described strategy, the target of the *nth* gene is made by a single-guide RNA (sgRNA) that will guide the Cas9 endonuclease. After the Cas9 protein cleaves the target site, the λ-red recombineering system repairs the double-stranded break (DSB) with a DNA template (dsDNA or ssDNA). Cells in which repair of the DSB does not occur will be unable to grow, thus allowing counter-selection of mutants. Subsequent modifications can be made using this strategy by curing the plasmid carrying the sgRNA and introducing another plasmid with a different

sgRNA. With optimization, these strategies could become the multiplexing tool that allows fast modification of LAB strains.

Material and Methods

Growth conditions and preliminary tests

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. *E. coli* DH5α was grown in 20 g/L of Luria-Bertani (LB) broth, *L. lactis* LMG19460 growth was done in M-17 (pH 7.0) supplemented with 20 g/L glucose and with the respective antibiotic. Antibiotic concentrations are specified in Table 2 but were also tested and optimized throughout this work, according to the necessary procedures. For growth of *L. lactis*, MRS media, LB broth and Elliker liquid medium (composed of tryptone 20 g/L, glucose 5 g/L, yeast extract 5 g/L, NaCl 4 g/L, sodium acetate 1.5 g/L,

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

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

Table 2. Growth conditions and antibiotic concentrations for the different strains and plasmids.

Plasmid	Strain	Antibiotic concentrations	Growth conditions
pTRKH3	E. coli DH5α	500 µg/mL erythromycin	37°C, 250 rpms
	L. lactis LMG19460	5 µg/mL erythromycin	30°C, 100 rpms
pKD46	E. coli DH5α	100 µg/mL ampicillin	30°C, 100 rpms
	L. lactis LMG19460	1.5 µg/mL ampicillin	
pKD13	E. coli DH5α	25 µg/mL kanamycin	37°C, 250 rpms
pCP20	E. coli DH5α	25 µg/mL ampicillin	30°C, 100 rpms
	L. lactis LMG19460	1.5 µg/mL ampicillin	
pCas9cr4	E. coli DH5α	50 µg/mL chloramphenicol	37°C, 250 rpms
	L. lactis LMG19460	1.5 µg/mL chloramphenicol	30°C, 100 rpms
pCas9cr4_pAMβ1_ery	E. coli DH5α	500 µg/mL erythromycin	37°C, 250 rpms
	L. lactis LMG19460	5 µg/mL erythromycin	30°C, 100 rpms
pKDsgRNA – p15 (and derivatives)	E. coli DH5α	50 µg/mL spectinomycin	30°C, 100 rpms
	L. lactis LMG19460	1000 µg/mL spectinomycin	

Table 3. 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. 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
sgRNA_nth_F	**gcagaagcctacggaattcc**GTTTTAGAGCTGTGAAAACAGC	2,868 bp
pKDsgRNA-frag1rev	TCGAGCTCTAAGGAGGTTATAAA	
sgRNA_nth_R	**ggaattccgtaggcttctgc**GTGCTCAGTATCTCTATCACTGA	4,434 bp
pKDsgRNA-frag2fwd	CCAATTGTCCATATTGCATCA	
sgRNA_conf	AGCTTTCGCTAAGGATGATTT	-
pCas9cr4_assembly_F	aat***ggcgcgcc***TGCTTGGATTCTCACCAATAAAAAAC	6,770bp
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	

ascorbic acid 0.5 g/L at pH of 6.8), were also 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. *L. lactis* LMG19460 was grown in solid regeneration medium[11] (composed of 10 g/L tryptone, 5 g/L yeast extract, 200 g/L sucrose, 10 g/L glucose, 25 g/L gelatin, 15 g/L agar, 2.5 mM $MgCl_2.6H_2O$, 2.5 mM $CaCl_2$), supplemented with the appropriate antibiotic and incubated at 30°C.

Growth limits of *L. lactis* LMG19460 were evaluated in solid medium at different temperatures: 30°C (control), 37°C, 39°C, 40°C, 41°C, 42°C and 43°C.

E. coli DH5α were turned chemically competent[12] or electrocompetent[13] and transformed accordingly. *L. lactis* was turned electrocompetent with glycine[11], an attempt at using NaCl in high concentration was also made[14].

To confirm the identity of the cells used throughout this study a molecular protocol was used, based in Salbi *et al.* (2014)[15], targeting the *hisG* gene by PCR amplification.

Reisch & Prather (2015)[10] strategy

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[16] and SnapGene[17] 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[18]. The guide RNA was selected to target the *nth* gene using an online software (CRISPR MultiTargeter[19]). Potential off-target sites were verified using the online software Cas-OFFinder[20]. To clone the sgRNA into the pKDsgRNA-p15 plasmid a Circular Polymerase Extension Cloning (CPEC) strategy was used, following the Reisch & Prather (2017)[21] protocol, and the primers in Table 3 (sgRNA_nth_F/pKDsgRNA-frag1rev for fragment 1 and sgRNA_nth_R/pKDsgRNA-frag2fwd for fragment 2).

The pCas9cr4 plasmid needed optimization for use in *L. lactis* and cloning of pAMβ1 origin of replication and the erythromycin resistance gene

(*erm*) was attempted by DNA ligation and Gibson Assembly, in parallel, using primers (Table 3) that allowed both approaches (pCas9cr4_assembly _F/R for pCas9cr4 amplification and linearization, pAMβ1_assembly_F/R for pAMβ1 amplification and pAMβ1_ery_assembly_F/R for pAMβ1 + *erm*).

Datsenko & Wanner (2000)[7] strategy

To confirm knockout of the *nth* gene in the cells provided by and obtained in Duarte (2018)[9], first the presence of pKD46 plasmid in *L. lactis* LMG19460 was confirmed (by amplification of the *bla* gene present in the plasmid), using the primers Amp_pKD46_F/R, in Table 5. Positive colonies were tested for the integration of the kanamycin cassette using conf_nth_F/R primers. Due to dubious results on the identity of the cells, the strategy was restarted in the present work.

L. lactis LMG19460 cells were transformed with pKD46 using 3 or 4 electric pulses, 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. Confirmation of transformants was done using the primers Amp_pKD46_F/R and pDNA (extracted from the resulting colonies) as template.

The kanamycin resistance cassette was amplified from pKD13 plasmid, using the primers KO_nth_F/R. *L. lactis* LMG19460 cells containing the pKD46 plasmid were turned electrocompetent in the presence of 1 µg/mL of ampicillin and L-arabinose, and transformed with 500 ng of DNA previously incubated with TTAB 30 mM for 30 min at room temperature and 3 or 5x electric pulses. , 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. Confirmation of integration of the kanamycin cassette was done using gDNA as template and Conf_nth_F/R primers.

Results and discussion

The preliminary tests served to evaluate some of the necessary conditions to apply the genetic engineering strategies in *L. lactis* LMG19460. 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, however, due to the stress of some procedures (for example, the electrocompetence protocols) the antibiotics were used at half the concentration.

The Datsenko & Wanner strategy requires that the FLP recombinase expression is induced by a temperature of 43°C. Preliminary tests to assess cell viability in this condition were done, showing that the strain is only able to grow up to 41°C.

Reisch & Prather (2015)[10] strategy

For knockout of the *nth* gene, this strategy requires first the introduction of a plasmid carrying the *Cas9* gene under control of the tet promoter being repressed by TetR (pCas9cr4), then the plasmid carrying the sgRNA targeting *nth* and the *λ-Red* genes, also controlled under the tet promoter, and that will integrate a ssDNA into the target region on the genome.

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 (oligo: 5’ TGGACCATGGAATAGTTAATAGAGATAATGG ACGTGCACGAATTAAACTTTCAAAACGTTTGA

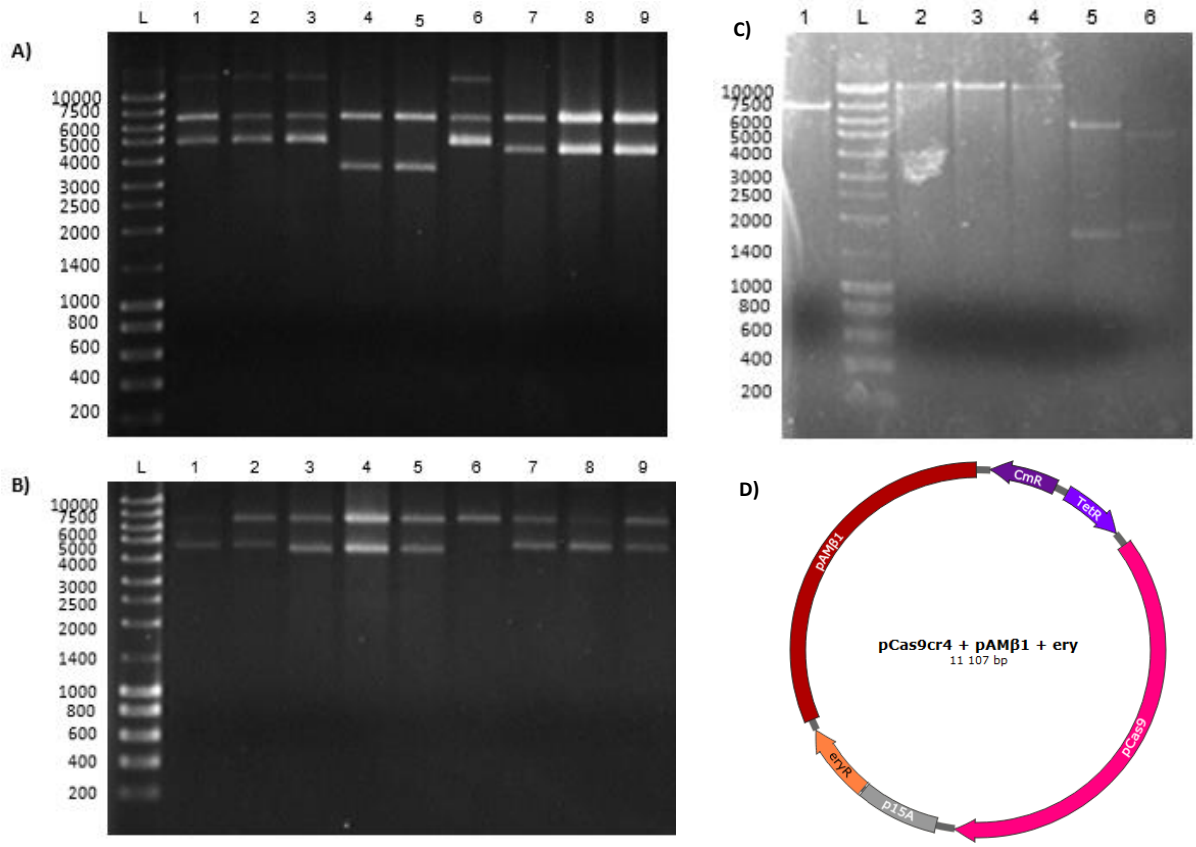

Figure 1. Double digestion with SgsI and BsrGI of pDNA obtained from the colonies resulting from the cloning process. **A)** from Gibson assembly, lanes: Nzyladder III (Nzytech), 1-3) 1 h reaction with pAMβ1 fragment, 4-6) 3 h reaction with pAMβ1 fragment, 7) 1 h reaction with pAMβ1 + eryR fragment, 8-9) 3 h reaction with pAMβ1 + *erm*; **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) 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. **C)** 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. **D)** Desired construction of pCas9cr4 + pAMβ1 + *erm* fragment.

AAGCAGTAGCTAATTAT 3') This sequence was tested *in silico* for secondary structures showing ΔG < - 12.5 kcal/mol. showing no need of shifting the sequence.

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. Since it is unconfirmed if *L. lactis* LMG19460 has the necessary proteins, cloning of a broad host range origin of replication from Gram-positive bacteria, such as pAMβ1 into this plasmid, should allow the replication inside the lactococcal strain. To allow for future controlled experiments if the need of two different selection markers arises, the resistance to erythromycin gene (*erm*) was also added to pCas9cr4 plasmid. The *L. lactis* strain is sensitive to low concentrations of erythromycin and this result is common to other *Lactococcus* and *Lactobacillus* species[22]. Optimization of this plasmid would be an advantage for application of this strategy in a variety of LAB hosts. Cloning was then attempted by restriction enzymes (SgsI and

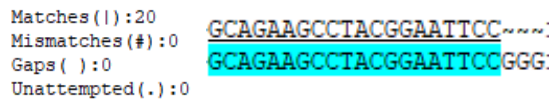

Figure 2. Alignment of the single-guide RNA (underlined) targeting *nth* gene from the *L. lactis* LMG19460 genome (blue), next to a PAM sequence (5' GGG 3') to the done in APE software.

BsrGI) and subsequent ligation with T4 ligase and Gibson assembly. The resulting constructions retrieved from *E. coli* DH5α were digested with SgsI and BsrGI (Figure 1A and B), and one from each cloning strategy showing the expected pattern was sequenced by Stabvida. The results showed that both attempts with the pAMβ1 + *erm* insert and pAMβ1 only insert, had mutations in pAMβ1. To overcome mutations created in amplification of the fragments, perhaps it is best to use a strategy that requires smaller fragments. The pCas9cr4 + pAMβ1 + *erm* plasmid (carrying mutations in pAMβ1) was introduced in the *L. lactis* strain and was able to replicate, however, when extracted and digested with BamHI, BglII and Alw44I (supposed to linearize the plasmid) to evaluate the total length of the pDNA, it showed an unexpected pattern, different than the one from pDNA recovered from *E. coli* (Figure 1C). The plasmid recovered from *L. lactis* was sequenced, confirming that both *erm* and pAMβ1 were not lost. This pattern suggests a loss of 3,500-4,000 bps of the original plasmid, which happens to be similar to the size of the *Cas9* gene (4,104 bp), it is then possible that the 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 instability[23]. 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.

The *in silico* design of sgRNA was done so that it 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[20]. Cloning of the sgRNA into the pKDsgRNA-p15 plasmid was done with CPEC and the resulting plasmid was sequenced using the sgRNA_conf primer (Table 3). Alignment to the *L. lactis* LMG19460 genome shows that the cloned sgRNA targets correctly the *nth* gene (Figure 2). 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.

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.

Datsenko & Wanner (2000)[7] strategy

After evaluation of the cells obtained by Duarte (2018)[9] using the Datsenko & Wanner (2000)[7] strategy, it was necessary to retry the knock-out

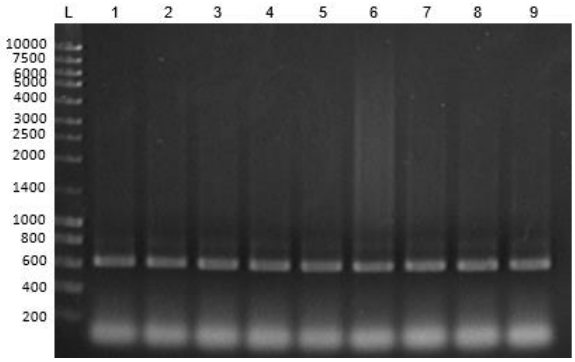

Figure 3. Lanes: Nzyladder III (Nzytech); PCR amplification with Ampl_pKD46_F/R primers targeting the *bla* gene (614 bp) of pDNA extracted from L. lactis LMG19460 after transformation in the following conditions: 1) with 30 min TTAB 30 mM incubation, 100 ng of pKD46 from E. coli DH5α. 3x pulses; 2) 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.

strategy of the *nth* gene, due to dubious identity of the cells. Firstly, the pKD46 plasmid was transformed into electrocompetent *L. lactis* LMG19460 cells. This plasmid has a low copy number and carries an antibiotic resistance gene to ampicillin to which *L. lactis* is highly sensitive. This makes effectiveness of the process of transformation 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 replication of 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[24]. 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[25]. Confirmation was done by PCR amplification using Amp_pKD46_F/R primers and

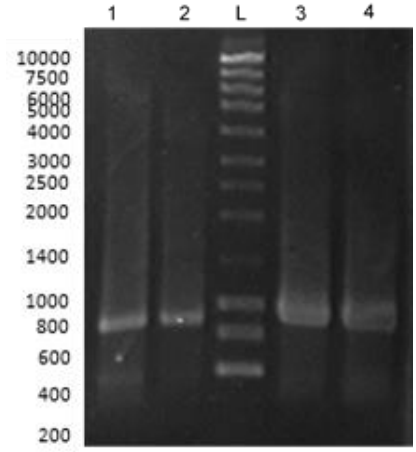

Figure 4. PCR amplification targeting the *hisG* gene and *nth* gene regions in the genome. Lanes: 1-2) amplification of the *hisG* gene from the gDNA obtained from two different colonies (933 bp); Nzyladder III (Nzytech); 3-4) amplification of the *nth* gene from the gDNA obtained from two different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

pDNA as template, all variables showed a positive result (Figure 3).

A positive colony was turned electrocompetent 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[9]. 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. Elliker did not result in any colonies and the other media allowed for growth that was tested both by colony PCR and using gDNA as template with the KO_nth_conf primers. When gDNA was extracted, the *hisG* gene was also amplified to test the identity of the cells (positive result shows a 933 bp fragment, indicating the presence of *L. lactis* LMG19460). The results showed that the desired strain was present, however, as wild-type, showing no integration of the kan-cassette into the genome. The electrocompetence protocol used as

standard for *L. lactis* LMG19460 in the laboratory uses glucose and sucrose in the process, along with gycine. 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 high salt concentration protocol for electrocompetence[14], 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. 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. 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, reported applications of this strategy in LAB, have used other antibiotic resistance cassettes, to which the bacteria are more susceptible[8].

Conclusion

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[26]. 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[27].

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

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