APPLICATION OF CONJUGATION TO PRODUCE GENETICALLY MODIFIED LACTOBACILLI

MASTER'S DEGREE IN MOLECULAR BIOLOGY AND BIOMEDICINE

2020/2021

IRENE MARTÍNEZ ROMÁN

DIRECTOR: MATXALEN LLOSA

IBBTEC

ABSTRACT

Lactic acid bacteria play a crucial role in the production of dairy food products, as well as in human and animal health as part of the microbiota. In particular, lactobacilli strains are well-known for their properties as probiotics and hence, they constitute an ideal candidate for therapeutic molecules delivery in humans. Despite their potential in the industry and biomedical applications, the genetic modification of this group is challenging due to their low transformation efficiency and complex diversity, especially in wild type strains. Recently, bacterial conjugation from *Escherichia coli* to lactobacilli strains has been successfully performed, opening a wide range of new possibilities; however, conjugation frequencies obtained were low. In this work we have tried to optimize this conjugation protocol to lactobacilli strains. First, several parameters of the protocol were modified such as the growth phases and the ratios between donors and recipients. During these experiments we have not seen an increase of the conjugation frequency; in fact, a decrease was observed under some conditions. We have also studied the effect of DNA methylation in bacterial conjugation frequencies using as donor an *E. coli* strain with a mutation in the DNA-cytosine methyltransferase system, in order to avoid restriction systems in the recipient. No increase in the transfer frequency of unmethylated DNA was observed. Finally, we have designed two different plasmid systems in order to combine bacterial conjugation with homologous recombination techniques to perform specific gene modifications in the recipient cell.

INDEX

1. INTRODUCTION

1.1 Lactic acid bacteria (LAB)

Lactic acid bacteria are a heterogeneous family of microorganisms characterized by the production of lactic acid as the main metabolism product. These microorganisms are mainly Gram-positive, anaerobic, non-sporulating and acid-tolerant bacteria. They can be naturally found in a wide range of habitats including dairy fermented meat and vegetables, plant environments and water (Klaenhammer et al., 2005). Based on their capacity to ferment carbohydrates, LAB can be classified as homofermentative or heterofermentative. Homofermentative lactic acid bacteria produce lactate from glucose whereas heterofermentative LAB yield lactate, ethanol and carbon dioxide (Ayivi et al., 2020).

LAB constitute an important component of the human gastrointestinal tract, therefore contributing to human health and being commonly used as probiotics. In addition, their metabolic products make them very important microbes in industrial food fermentations. Traditionally, LAB have been extensively used as starter cultures for dairy fermentations, leading to the widespread human consumption and to generally recognized as safe (GRAS) status by the FDA. Consequently, LAB, in particular lactoccoci and lactobacilli, have gained interest as mucosal delivery vehicles in order to introduce therapeutic molecules (section 1.1.1).

The genus *Lactobacillus* was the largest within the LAB. It was exceptionally large and diverse, including 261 species very different at the phenotypic, ecological and genotypic level. The exceptional diversity of this genus was one of the reasons for their unclear taxonomy, impeding the correlation of strains phylogenetic relationships with their physiological properties (Zheng et al., 2015). Recently, this genus has been revisited and a new classification into 25 genera has been stablished, thus reflecting the great biodiversity among the species previously grouped as *Lactobacillus*. In this work the term "lactobacilli" will be used to designate all organisms classified as *Lactobacillaceae* until 2020 (Zheng et al., 2020). Lactobacilli species are Gram-positive, homofermentative, thermophilic and non-spore-forming rods. They ferment a wide spectrum of carbohydrates and have the ability to ferment extracellular fructans, starch, or glycogen (Zheng et al., 2020)

1.1.1 Biotechnological and biomedical applications

LAB play an important role in human and animal health as they are involved in homeostasis processes, provide protection against pathogenic bacteria and stimulate the immune system. LAB

beneficial effects on human health have caused *Lactobacillaceae* and *Bifidobacterium* to be the most commonly used as probiotics (Isolauri et al., 2004). Probiotic products are live microorganisms which, when administered in adequate amounts, have a beneficial effect on the host. They are proved to reinforce gastrointestinal health by shortening rotavirus diarrhea, reducing recurrence colorectal cancer and decreasing the risk of infections in healthy subjects. Also, they can relieve the lactose intolerance symptoms, as the fermented milk products produced are tolerated by lactose maldigesters (Klaenhammer et al., 2005; Ouwehand et al., 2002).

Lactobacilli strains constitute part of the normal microbiota where they colonize the gastrointestinal and urogenital tracts and maintain the intestinal integrity (Isolauri et al., 2004). Hence, they are able to cross the mucosa barrier due to their tolerance to temperature, high pH, bile salts and high alcohol concentrations. For these characteristics, they have been proposed as ideal live vectors for the *in situ* production of therapeutic agents in the human mucosae (Bosma et al., 2017; Cano-Garrido et al., 2015). Up to now, lactobacilli have been used as prophylactic agents and adjuvants against several diseases (Mays and Nair, 2018).

In addition to their biomedical applications, these organisms play a crucial role in the production of dairy food products and are extensively used as fermentation starter cultures (Bernardeau et al., 2008). The fermentation process consists on the formation of lactic acid from carbohydrates resulting in a rapid acidification of the food. This acidification acts as a natural antimicrobial against pathogens and other microorganisms, resulting in a tool to ensure the safety and quality of food products. The use of lactic acid bacteria in food preservation is called bio-preservation and is a natural alternative for controlling unwanted microbiota in food (Ayivi et al., 2020). Furthermore, they are often used to improve flavour and texture of food as they are responsible for the organoleptic properties of the food products (Isolauri et al., 2004)

The industrial applications and the role of lactobacilli in human health makes them one of the most important bacteria taxa economically (Zheng et al., 2015). However, lactobacilli biotechnological and biomedical applications are limited, since genetic tools for their progress are still underdeveloped (Bosma et al., 2017).

1.1.2 Introduction of exogenous DNA in LAB

Given the interest of LAB and their many applications, it is no surprise that this group was a pioneer for the study of the development of genetic tools. The first studies were performed on *Lactococcus lactis* due to its importance in daily fermentations. This formed the basis for hundreds of subsequent studies that have been carried out until today. Apart from this, many other LAB are highly transformable and are considered to be genetic models systems (de Vos, 2011). However, in some LAB strains, transformation efficiencies are very low or, as in some wildtype strains, transformation is not even feasible.

The first step in order to accomplish genetic modification is the introduction of DNA, which can be achieved by transformation methods such as electroporation, conjugation, phage transduction and natural competence (**figure 3**). The most used method for the introduction of foreign DNA in LAB is electroporation. Its simplicity and efficiency make it suitable to introduce foreign DNA into a wide range of bacteria. For this reason, several LAB strains have been successfully transformed by generalised electroporation protocols. However, efficiencies in LAB are highly variable, as large differences have been reported even among different strains of the same species. As an example, efficiencies from several strains of *Latilactobacillus sakei* range from 10^2 transformants/μg in CTC335 to 10^6 in 64F (Wang et al., 2020). Moreover, transformation of certain wildtype lactobacilli using this method is difficult or not even feasible (Börner et al., 2019). This limitation highlights the importance of exploring new approaches in order to introduce DNA efficiently into LAB, such as bacterial conjugation. This method has been described naturally among LAB *in vivo* in our microbiota, including LAB as recipients. Also, it has been reported from *E. coli* to *Bifidobacterium,* and recently to lactobacilli (section 1.2.3).

Apart from bacterial conjugation, LAB transformation can be achieved by other natural methods, such as phage transduction and natural competence. Bacteriophage transduction is formed by viruses which infect bacterial cells. It has been broadly studied in LAB as it responsible of many fermentation failures in dairy industrial processes. Genes associated with antibiotic resistance, sugar fermentation or antibiotic resistance have been transferred between LAB by this method. Remarkably, it has been observed in poor genetically accessible lactobacilli such as *Lactobacillus dellbrueckii*, and even between species, as *L. lactis* and *Streptococcus termophilus*. However, host-specificity is determined by the phage-encoded receptor binding proteins which associate to the correspondent receptor in the host surface. Thus, leading to a phage-host recognition that could limit the genomic mobilization potential (Bron et al., 2019).

Natural competence is another natural method which is based on the entry of exogenous single-stranded DNA into the cell mediated by a native DNA uptake machinery. Among the industrial LAB, this mechanism has been specifically studied for *Streptococcus thermophilus*. In lactobacilli, several strains were reported to have the complete DNA uptake machinery, although some of its genes appeared to be mutated or disrupted (Bron et al., 2019). This method has been described as another interesting approach for non-genetically accessible LAB transformation (Börner et al., 2019).

LAB transformation is partly restricted by their thick peptidoglycan layer, characteristically of gram-positive cell walls, thus interfering in the foreign-DNA entry and causing low transformation efficiencies. In some cases, this entry is also impeded by the restriction barriers that are present as a defence inside the bacteria. This restriction-modification (RM) systems recognize and distinguish self-DNA from foreign DNA. Therefore, DNA with a different methylation pattern is recognized as non-self and cleaved, not allowing its entry into the cell, and therefore, its genetic modification (Mays and

Nair, 2018; Spath et al., 2012; Vasu and Nagaraja, 2013). In order to bypass this barrier and allow the DNA entry, several studies have reported successful transformations with non-methylated DNA. Specifically, in *Staphylococcus aureus* and *S. epidermidis*, two strains that were previously untransformable, a successful transformation has been performed by using a mutant deficient in methylase as a donor strain, thus introducing non-methylated DNA and bypassing the restriction barrier (Costa et al., 2017; Monk and Foster, 2012; Monk et al., 2012). Moreover, in some *Bifidobacterium* and lactobacilli strains, similar restriction mechanisms have been described and, specifically in *Bifidobacterium adolescentis, Lactococcus lactis (Yasui et al., 2009)* and *Lactiplantibacillus plantarum* (Spath et al., 2012), transformation efficiencies were improved by electroporating non-methylated DNA.

Natural mechanisms of DNA entry are acceptable methods for genomic modification in the European Union in order to obtain GRAS microorganisms for the human consumption (Derkx et al., 2014). However, these modifications are mostly focused on the development of food-grade microorganisms, instead of optimizing the mutagenesis procedures, as the strains obtained are considered genetically non-modified organisms. The main limitation of these methods is that they can introduce unwanted mutations, on the contrary to targeted methods, which allow accurate targeted mutations (Derkx et al., 2014). Targeted genetic modification techniques include homologous recombination approaches, site-specific integration and CRISPR-Cas, and will be described in detail in the 1.3 section.

1.2 Bacterial conjugation

Bacterial conjugation is a mechanism of horizontal DNA transfer from a donor to a recipient bacterial cell which requires physical contact though a protein complex (Llosa and de la Cruz, 2005). This mechanism generates genetic variability in bacteria and, as a consequence, it is the most important mechanism of spreading antibiotic resistance genes and virulence factors (De la Cruz and Davies, 2000). Conjugation allow the efficient transfer of any DNA molecule with an *oriT*. Moreover, is a promiscuous process which can happen between distant taxonomical bacteria and even to eukaryotic cells (Waters, 2001). These characteristics make conjugation a potential biotechnological tool for customize *in vivo* DNA delivery.

The conjugative system is composed by the assembly of three different modules that form a complete system (Llosa and de la Cruz, 2005):
- The type IV secretion system (T4SS), which forms a channel from the donor to the recipient cell to deliver the DNA. This transmembrane channel is a multiprotein complex formed by about 10 transporter proteins from the T4SS family. Some members of this family are also involved in pathogenesis, introducing virulence factors into mammalian target cells.
- The relaxosome or substrate *selector*, composed by a relaxase, an *oriT* and one or more accessory nicking proteins. The relaxase specifically cleaves and attaches covalently to the

oriT in the DNA strand to be transferred. At the end of the process, this protein religates the cleaved strand. It is also called the selector due to its specificity for each plasmid system.

- The coupling protein (CP), which puts in contact the relaxosome and the channel, approaching both parts of the transfer machinery.

1.2.1 Bacterial conjugation cycle

The general scheme for the bacterial conjugation cycle can be differentiated in several steps (**Figure 1**):

1) First a donor cell and a recipient cell get in contact. This is allowed by a protein structure formed by the donor cell and called the conjugative pilus. The contact is mediated by proteins of the mating pair formation (*mpf*) complex.

2) The enzyme called relaxase (R) recognizes its target, the *oriT* in the DNA to be transferred. It performs a single-strand cleavage and binds covalently to the 5' end. Then the double-stranded DNA unwinds and a single-stranded molecule appears in order to be mobilized into the recipient cell.

3) The relaxase-ssDNA complex is then transferred through the channel into the recipient cell helped by the CP.

4) Once it is inside the recipient cell, the single-stranded DNA is recircularized, converted into double-stranded DNA and supercoiled, in order to stablish in the recipient cell.

5) When the process is finished, the cells split up and entry-exclusion determinants are expressed in the recipient cell to prevent more copies of the same plasmid to enter again. The recipient cell can now act as a donor and transfer the DNA starting the conjugation cycle again.
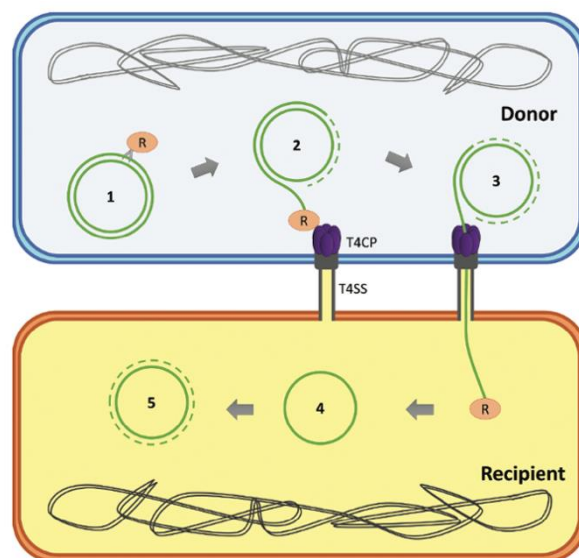

Figure 1. General scheme for bacterial conjugation cycle. Taken from (Getino and de la Cruz, 2019).

1.2.2 Plasmids R388 and RP4

Transmissible plasmids can be classified depending on their mobilization capability in conjugative, which are self-transmissible, or mobilizable, which can only be transmitted with a helper plasmid (Francia et al., 2004). The majority of plasmids studied correspond to gram-negative bacteria, and specifically in *Enterobacteriaceae* family six incompatibility groups of conjugative plasmids have been stablished: IncF, IncI, IncW, IncN, IncP and IncX (Couturier et al., 1988).

Plasmids can also be grouped according to the variety of host bacteria that they can settle on, being broad host range plasmids (IncN, P, W) or narrow host range plasmids (IncF and I). Broad host range plasmids can replicate in a wide variety of bacteria, in contrast, narrow host range plasmids only in a limited number of really similar species. Moreover, an additional classification can be done depending on the type of pilus. Bacterial pilus can be flexible, allowing the bacteria to conjugate on liquid and solid media, or stiff, only allowing the bacteria to conjugate on solid media (Grohmann et al., 2003).

Regardless of all these plasmid classifications and different properties, all plasmids share certain characteristics for the conjugation to happen. They all synthesize a mechanism to promote cell contact, such as the conjugative pilus, they need DNA-processing enzymes in order to initiate the DNA transfer, a mechanism for ensuring the establishment of the incoming plasmid in the recipient cell, a regulatory system for transfer control and induction and an origin of transfer (*oriT*), a short sequence to be recognized in order to be transferred (Zechner et al., 2000).
The two main mobilizable plasmids used in this work are R388 and RP4.

<u>Plasmid R388</u>
Plasmid R388 was first isolated from *E.coli* and described in 1972 (Datta and Hedges, 1972). It is a conjugative plasmid of broad host range which forms part of the IncW incompatibility group and it confers resistance to trimethoprim and sulphonamides (Avila and de la Cruz, 1988). Its pilus is stiff and thin, so conjugation can only be performed on solid media (Bradley, 1980).

Conjugative plasmids of the IncW group have the smallest genome size among natural plasmids studied. Plasmid R388 contains 33kb and 43 ORFs and it can be organized in five functional regions based in functional assignment (**Figure 2**). Genes belonging to these regions can be classified in two major sectors: one corresponds to the basic functions of survival and the other to conjugation. Genes from the first region are grouped in three modules: replication, stable inheritance and establishment. The modules related with conjugation are Mpf (mating pair formation), which contains the genes responsible for the T4SS synthesis and assembly and Dtr (DNA transfer replication) which contains the genes necessary for DNA processing and mobilization (Fernández-López et al., 2006).
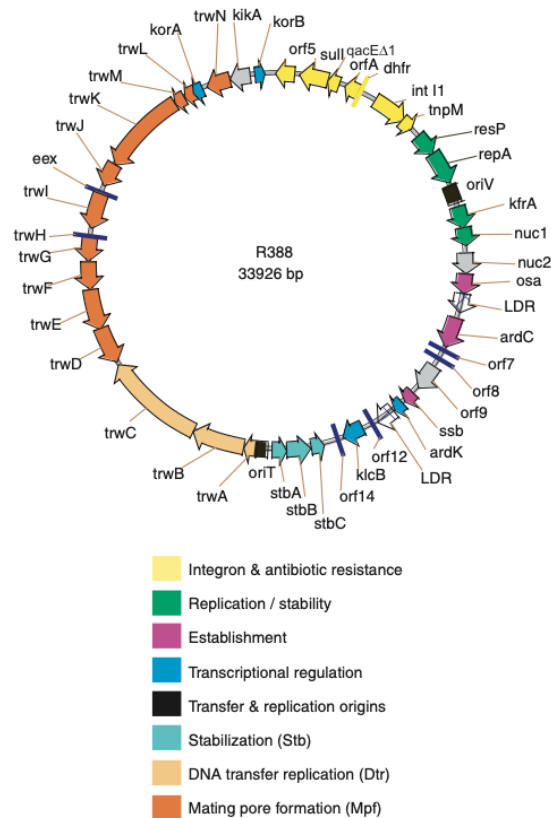
Figure 2. Genetic map of the R388 plasmid. In the figure is showed the genetic disposal of the R388 plasmid. The sequence is classified in different colors according to the different functional modules specified in the code below (see text for details). Figure from (Fernández-López et al., 2006)

Plasmid RP4

Plasmid RP4 is a broad host range conjugative plasmid which forms part of the IncPα incompatibility group. Its size is 60kb long and the conjugative functions are encoded by two main regions of 15,7 kb, Tra1 and Tra2. (Haase et al., 1995). RP4 plasmid has been the most used in the lab to send DNA between bacteria taxonomically distant, including eukaryotes.

1.2.3 Conjugation from Gram-negative to Gram-positive bacteria

Bacterial conjugation is promiscuous; not only it can happen between different genera, but it can also happen between Gram-positive and Gram-negative bacteria (Trieu-Cuot et al., 1988). DNA transfer can occur naturally from bacteria to the nucleus of plant cells in certain species of *Agrobacterium* through a process highly related to conjugation (Zupan et al., 2002). Moreover, under laboratory conditions, conjugation has been observed between different kingdoms: from bacteria into yeast (Heinemann and Sprague, 1989), plants (Buchanan-Wollaston et al., 1987) and even mammalian cells (Waters, 2001).

Bacterial conjugation has also been described naturally among LAB *in vivo* in our microbiota, thus including LAB as recipients (Aviv et al., 2016). Conjugative DNA transfer from *E. coli* to *Bifidobacterium* has been reported (Dominguez and O'Sullivan, 2013), and recently, conjugation from *E.coli* to lactobacilli has been described (Samperio et al., 2021). Conjugation to lactobacilli was performed as an alternative to electroporation due to the existent limitations for the introduction of DNA in these organisms. The plasmids used for it were R388 and RP4 and recipient strains were successfully transformed, including lactobacilli wildtype strains which were not amenable to electroporation, such as *Lentilactobacillus parabuchneri,* and even *Staphiloccocus epidermidis* from the researcher contamination. However, the conjugation frequencies obtained were low and the conjugation protocol still needs to be optimized. (Samperio et al., 2021).

1.3 Targeted genetic modification in bacteria

Untargeted natural methods have been widely used in the industry in order to obtain food-grade-microorganisms, as these are considered genetically non-modified. However, these methods are described to produce spontaneous mutagenesis. For this reason, the development of targeted genomic modification methods is essential for successful customized genetic modification. Nevertheless, methods leading to the integration of foreign DNA in LAB are considered problematic from the consumers and the regulatory legislative view. And, for this reason, the industry has been focused on avoiding the usage of these methods and instead use the untargeted ones (Plavec and Berlec, 2020).

Genome-engineering tools in LAB can be achieved through plasmid-encoded expression systems which are used for gene cloning, expression and secretion of LAB. However, plasmids instability is the major source of disruption in the production of fermented products (Plavec and Berlec, 2020), as well as phage infection. In order to avoid these problems, several versatile cloning vehicles based on endogenous replicons and selection markers have been developed. Specifically, the construction of vectors which allow the direct insertion of DNA sequences into a precise site in the LAB genome, therefore stabilizing the foreign DNA (Alvarez *et al.*, 1998). Chromosome modifications can be performed by phage integration systems, homologous recombination approaches or, by more recent genome-engineering tools, such as CRISPR-Cas (Plavec and Berlec, 2020).

1.3.1 Site-specific integration

Traditionally, chromosomal integration strategies have been achieved through phage integration systems and homologous recombination-based systems. Lysogenic phages naturally perform site-specific integration into the host DNA, usually without inactivating host genes.

Therefore, vectors encoding these phage integration system are very convenient in foreign-DNA stabilization (Alvarez et al., 1998). Site-specific integration occurs through a recombination step between the phage attachment site, *attP,* and the host attachment site, *attB.* The recombination process is catalyzed by several proteins. The site-specific recombinases can be grouped in two major families: the Int and the resolvases or invertases. The Int family catalyze the recombination between sites in the same or separate DNA molecules, therefore allowing the insertion at the *attB* site. The resolvases or invertases catalyze recombination between sites only in the same DNA molecule. *b*-Recombinase is an enzyme from this family that allow intramolecular deletions and inversions of sequences located between two 90-pb target sites, also called *six* site (Martín et al., 2000).

A specific example of this methodology for lactobacilli has been performed based on the phage A2. A2 is a temperate phage which infects two lactobacilli strains: *Lacticaseibacillus casei* and *Lacticaseibacillus paracasei.* Based on the site-specific integration of this phage, a plasmid with an integration cassette was developed and showed to be functional in the natural hosts, other LAB and even unrelated Gram-negative bacteria such as *E.coli (Alvarez et al., 1998).* However, the site-integration of these cloning systems catalysed by the integrase cannot be used in commercial fermentation, as several unwanted sequences remain in the food product. In order to remove these sequences, the *b*-recombinase enzyme is used constituting a delivery and depuration system. Moreover, the system was validated by the cloning of the phage A2 repressor gene, *cI*, providing resistance to phage infection (Martín et al., 2000).

1.3.2 Homologous recombination approaches

Homologous recombination systems are the most frequently used for chromosomal insertions, deletions and gene replacements. They are based on the insertion of non-replicative vectors, typically suicide plasmids, into the target bacteria which contain sequences homologous to the insertion site. As an example, to knock out a gene on *E.coli* chromosome, a dsDNA cassette encoding antibiotic resistance is often used (Thomason et al., 2014). In addition, the development of this methodology has been focus on approaches that don't leave any marker or residual bases on the genome (Plavec and Berlec, 2020). This is based on the selection of a first recombinant by a selection marker, and then promoting the second recombination event which removes such marker. This allows the modification in vivo of the bacterial chromosome using different donor substrates, such as linear dsDNA, ssDNA, that have been introduced into the bacteria by electroporation.

Several strategies using homologous recombination techniques have been described for LAB. A suicide vector with an insert of *Lactobacillus johnsonni* was used in order to promote gene insertion into several lactobacilli species (Walker and Klaenhammer, 1994). Also, a thermosensitive plasmid with temperature-dependent replication was designed for the replacement of DNA

sequences from the genome of some lactobacilli strains (Plavec and Berlec, 2020). Another approach was done by the *upp* gene which encodes uracil phosphoribosyltransferase as counter selectable-marker for positive selection of *Lacticaseibacillus casei* (Song et al., 2014). All plasmids were introduced by electroporation.

1.3.3 CRISPR-Cas system

CRISPR-Cas systems are present in many bacteria and archaea as part of their adaptive immune system. CRISPR loci is formed by several DNA repeats separated by variable sequences called spacers (Horvath and Barrangou, 2010). In the immune response performed by this system, the target DNA is detected by the Cas proteins and a small portion of it is cleaved out. This sequence is then inserted in the CRISPR array, so it becomes a spacer. Therefore, when an invading genome of a virus or plasmid similar to this sequence enters into the cell, the system will recognize it, cleave it and inactivate it by a Cas nuclease (Makarova et al., 2020).

CRISPR-Cas-based gene modification tools are used for gene deletion, insertion and silencing in LAB. The most frequently used system is CRISPR-Cas9 which require a Cas9 protein and a single guide RNA (sgRNA). This RNA contains a sequence to bind to the target DNA in 5' and the Cas9 protein in 3'. By this mechanism several genes can be edited by shifting and introducing the sgRNAs designed. However, this system has several limitations, such as poor repair ability, off-target effect and high toxicity (Song et al., 2020).

CRISPR-Cas9-based genetic modification mechanisms have been successfully performed in some lactobacilli such as *Limosilactobacillus reuteri*, *Lacticaseibacillus casei, Lactiplantibacillus plantarum* and *Levilactobacillus brevis* (Song et al., 2020).
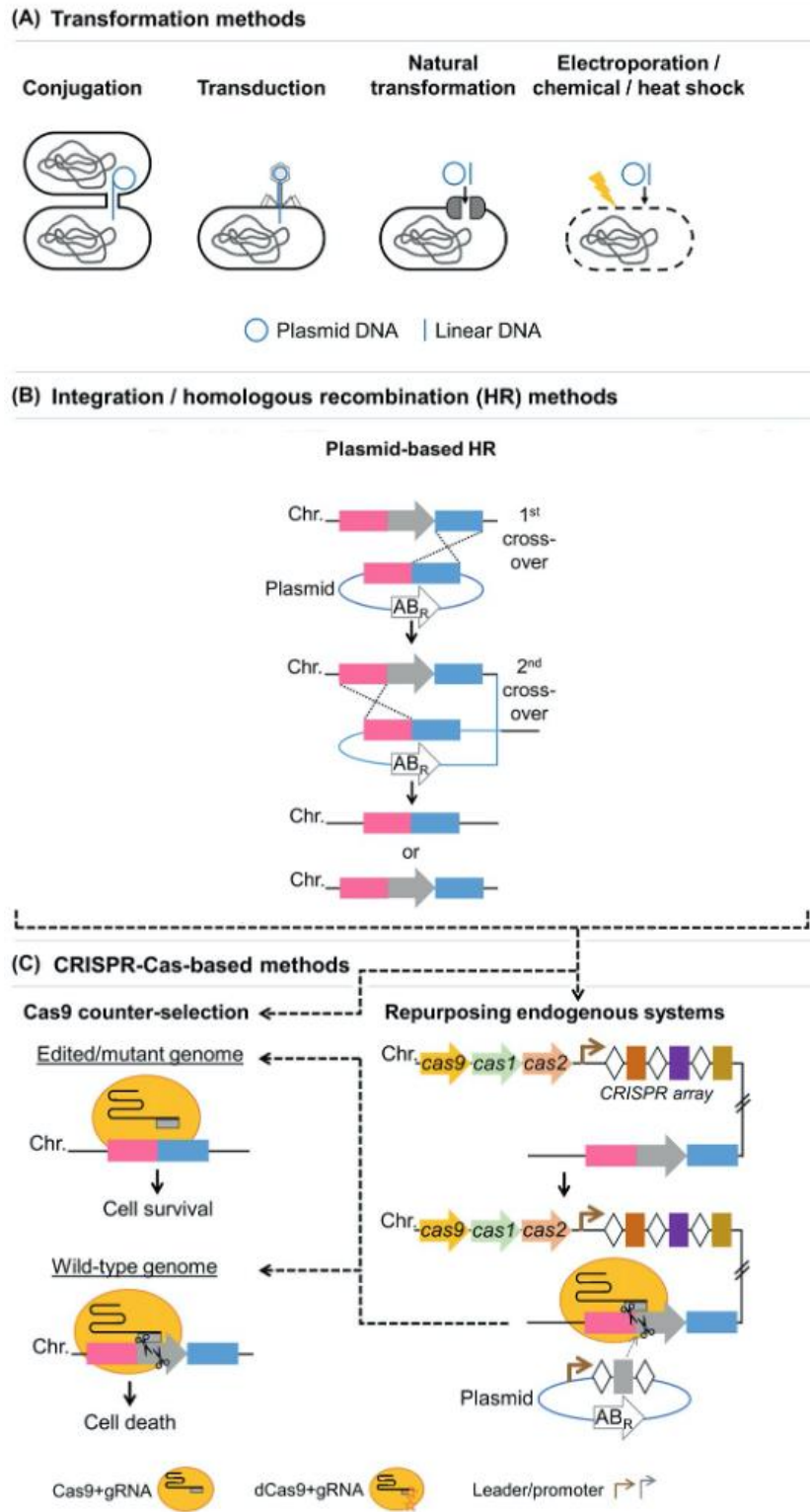
Figure 3. Transformation and genome editing methods currently available for LAB.
A) Transformation methods, including conjugation, transduction, natural transformation and electroporation.
B) Plasmid-based homologous recombination method using the native recombination machinery.
C) CRISPR-Cas based editing tools. Figure adapted from (Bosma et al., 2017).

2. OBJECTIVES

Lactobacilli include may species relevant for the food industry and their properties as probiotics make them attractive for therapeutic proteins delivery. Despite their many applications, scarce tools are available to accomplish genetic manipulation of this group. Bacterial conjugation from *E.* coli to lactobacilli was recently described in our lab as a new tool to introduce genetic modifications. However, conjugation frequencies obtained were low. In order to optimize the lactobacilli conjugation and combine it with genetic modification tools, the following objectives were established:

a. Optimization of the conjugation frequency from *E. coli* to lactobacilli.

- Modification of the physical parameters from the original conjugation protocol.

- Transfer of unmethylated DNA, using a strain deficient in Dcm methylase as a donor.

b. Design of a targeted genetic modification system for lactobacilli based on the introduction by conjugation of homologous recombination cassettes.

- Construction of mobilizable plasmids carrying a recombination cassette for lactobacilli.

- Introduction by conjugation and confirmation of expected mutations.

3. MATERIALS AND METHODS

3.1 Bacterial strains

The bacterial strains used, and their genotype are listed in **Table 1**.

Table 1. Bacterial strains used in this work.

Strain	Genotype	Phenotype	Reference
Echerichia coli			
D1210	SmR*; recA hspR hsdM rpsI lacIq*	Sm^R	(Sadler et al., 1980)
S17.1	SmR; *pro res mod1 RP4-2 Tet::Mu-Kan::Tn7*	Sm^R	(Simon et al., 1983)
DH5αT1^R	F- φ80lacZΔM15 Δ((lacZYA-argF) U169 recA1 endA1 hsdR17(rk-, mk+) phoA supE44 λ-thi-1 gyrA96 relA1 tonA.	Nx^R	(Killmann et al., 1996)
DC10B	SmR; mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL endA1 nupG Δdcm	Sm^R	(Monk et al., 2012)
Lacticaseibacillus paracasei			
Lacticaseibacillus paracasei BL23	Laboratory strain		(Mazé et al., 2010)

3.2 Plasmids

3.2.1 Published plasmids used in this work

Table 2. Published plasmids used in this work.

Plasmid	Phenotype	Description	Reference
pCOR48	Ap^R Em^R	Mobilizable shuttle vector *E. coli* and lactobacilli *oriV*, R388 *oriT*	(Samperio et al., 2021)
pCOR49	Ap^R Em^R	Mobilizable shuttle vector *E. coli* and lactobacilli *oriV*, RP4 *oriT*	(Samperio et al., 2021)
pCOR51	Ap^R Em^R	Suicide vector *E. coli oriV*, RP4 *oriT*	Coral González-Prieto
pEM94::oriT$_{RP4}$	Ap^R Cm^R	Mobilizable Shuttle vector *oriV* for *E. coli* and *ts oriV* for lactobacilli	Sara Samperio
pRL443	Tc^R Ap^R	RP4 derivate	(Elhai et al., 1997)
pSU711	Km^R Tp^R	R388Δ*oriT*	(Demarre et al., 2005)
pSU2007	Km^R Tp^R	R388 with km^R cassette in Su^R gene	(Martinez and de la Cruz, 1988)

3.3 MOLECULAR BIOLOGY TECHNIQUES

3.3.1 DNA extraction and purification

For DNA extraction and purification different kits were used depending on the starting material and the final product applications, following manufactures' recommendations.

3.3.1.1 Genome DNA extraction from lactobacilli

For total DNA extraction of lactobacilli, a colony from an MRS-agar plate was resuspended in 50 µl of TE buffer (10 mM Tris·HCL; pH 8.0, 1 mM EDTA). 50 µl of chloroform were then added and mixed thoroughly until the mixture is homogeneous. This was centrifuged 10 minutes at 4°C appearing three different phases. The upper one was collected carefully, as is the one containing the DNA, and used directly for PCR analysis (Samperio et al., 2021).

3.3.1.2 Plasmid DNA extraction

Plasmid DNA was extracted from *E. coli* with the GenElute Plasmid Miniprep kit (Sigma Aldrich).

For gel extraction and purification GeneJet Gel extraction kit (Thermo Scientific) was used. GeneJet PCR purification kit (Thermo Scientific) was used for PCR purifications.

The DNA concentration from the samples was measured with a Nano-Drop Spectrophotometer ND-1000 (Thermo Scientific).

3.3.2 Restriction enzyme digestion

Fast Digest restriction enzymes (Thermo Scientific) were used following the manufacturer's instructions. Reactions were performed in 20 µl, with DNA up to 800 ng and the recommended buffer at 37°C for 10-15 minutes. After the incubation time, the enzymes were inactivated by heating for 10 min at 60°C.

3.3.3 DNA electrophoresis

DNA and PCR products were analysed by agarose gel electrophoresis. Agarose was blend in TBE (Tri-HCL 45 mM, boric acid 45 mM, EDTA 0.5 mM, pH 8.2) to a final concentration of 1 % (w/v). Agarose gels were stained with SYBR Safe (Invitrogen) and a 6X Loading buffer (bromophenol blue 0.25 % (w/v), sucrose 40 % (w/v) in TBE) was used to dilute DNA samples. GeneRuler 1kb DNA ladder (Thermo Scientific) was used as a molecular weight marker. In order to perform electrophoresis, a horizontal BioRad electrophoretic device was used with constant voltage between 80-110 V. The gel was visualized with a Gel Doc2000 UV system, and images were analysed with Quantity One software (BioRad).

3.3.4 DNA sequencing

DNA samples were sequenced by Sanger DNA sequencing (STAB VIDA (Caparica, Portugal)).

3.3.5 PCR and primer design

For the amplification of DNA sequences used for cloning, PCRBIO HiFi Polymerase (PCRBIO Systems) was used. PCR reactions were set up to final volumes of 50 µl. A T100 Thermal Cycler (BioRad) thermocycler was used with the following program: 30 s of denaturation at 98°C; 35 cycles of amplification, including denaturalization step for 10 s at 98°C, annealing for 15 s at the correspondent annealing temperature of the primers and elongation at 72°C for 30 s/kb of the sequence; and the final extension of 5 min at 72°C.

For routine analyses and colony PCR, Kapa Taq DNA polymerase (Kapa Biosystems) was employed. A T100 Thermal Cycler (BioRad) thermocycler was used with the following program: 3 min of denaturation at 95°C; 35 cycles of amplification, including denaturalization step for 30 s at 95°C, annealing for 30 s at the correspondent annealing temperature of the primers and elongation at 72°C for 1 min/kb of the sequence; and the final extension at 72°C for 1 min/kb of the sequence.

After the reaction, samples were stored at 4°C for short periods of time or at -20°C for long-term conservation.

Primers were designed using Vector NTI 10.3 software (Invitrogen) with a length of 18-25 bp. In the primers designed to amplify insert fragments, a DNA tail with 30 bp of homology was attached to the 5' end Primers are listed in **Table 3**.

Table 3 Primers used for this work.

Amplification target	Sequence[1]
Sequence of homology *lacG₁*	
Tail pCOR51 + *lacG₁* F	5' ACTCATACTCTTCCTTTTTCAATTCGACCG**GGTAAGGTTTCCTTTGTGCC** 3'
Tail pEM::*oriT* + *lacG₁* F	5' GCTAAAATTGGTTATGCACGTGTCAGTAG**CGGTAAGGTTTCCTTTGTGCC** 3'
lacG₁ R	5' **CCTCCTGTTAAGTGTCTCAAAG** 3'
Sequence of homology *lacG₂*	
lacG₂ F	5' **GGCTATTTAGACAGCAAGG** 3'
Tail pCOR51 + *lacG₂* R	5' CAAAATTCGACCCGATTCACAAAAAATAGG**CCGCTACTGATTTTTGTAACCG** 3'
Tail pEM::*oriT* + *lacG₂* R	5' ATAGGCTAACGCCTGGCTTGGTTTTTCAGC**CCGCTACTGATTTTTGTAACCG** 3'
***em* from pCOR49**	
Tail *lacG₁*+ *em* F	5' AATCCAATCTTTGAGACACTTAACAGGAGG**GATCCCCGATCCGTCGAC** 3'
Tail *lacG₂*+ *em* R	5' TCCGATTCAATCCTTGCTGTCTAAATAGCC**GGCACACGAAAAACAAGTTAAGGG** 3'
pCOR51 amplification	
pCOR51 F	5' **CCTATTTTTTGTGAATCGGGTCG** 3'
pCOR51 R	5' **CGGTCGAATTGAAAAAGGAAGAG** 3'
pEM::*oriT* amplification	
pEM::*oriT* F	5' **GCTGAAAAACCAAGCCAGGC** 3'
pEM::*oriT* R	5' **GCTACTGACACGTGCATAACC** 3'
Isothermal assembly analysis	
51 F	5' **CTCTTCCTTTTTCAATTCGACCG** 3'
51 R	5' **CGACCCGATTCACAAAAAATAGG** 3'
RP4 F	5' **GGTTATGCACGTGTCAGTAGC** 3'
RP4 R	5' **GCCTGGCTTGGTTTTTCAGC** 3'
Em[R] F	5' **AGTACGGATATAATACGCA** 3'
Sequence *em*	
*em*_F	5' **AGTACGGATATAATACGCA** 5'

[1]Nucleotides annealing to the template during PCR amplification are shown in bold.

3.3.6 Cloning procedures: isothermal assembly

All clones were built by isothermal assembly (Gibson et al., 2009), a one-step cloning method based on the homology between the ends of the sequences to be assembled. This reaction includes a 5'exonuclease, a DNA polymerase and a DNA ligase **figure 4**. PCRBIO HIFI (PCR Biosystems) DNA polymerase was used to obtain the insert and vector fragments. The primers used for this PCR amplification contained a 30 bp tail homologous to the sequence to be assembled. In order to remove template plasmids, treatments with Fast Digest DpnI (Thermo Scientific) were performed in the PCR products at 37°C for 15 minutes. Afterwards, samples were purified with the GeneJet PCR purification kit (Thermo Scientific). For the isothermal assembly several ratios (inserts/vector) were used and for the reaction, a volume of 5 μl of inserts-vector mixture was combined with 15 μl of the Gibson buffer (1M Tris-HCl pH 7.5, MgCl$_2$ 2M; dNTPs 100mM; DTT 1M; 1,5 g of PEG 8000; NAD 100mM). The Gibson buffer also includes T5 exonuclease (Epicentre), Phusion polymerase (Thermo Scientific) and Taq ligase (New England Biolabs). Isothermal assembly reaction was performed at 50°C for 1h. Then, samples obtained were microdialyzed for 25 min using a 0.05 μm wide pore nitrocellulose filter (Millipore GS) and electroporated in electrocompetent cells (Section 3.4.3).
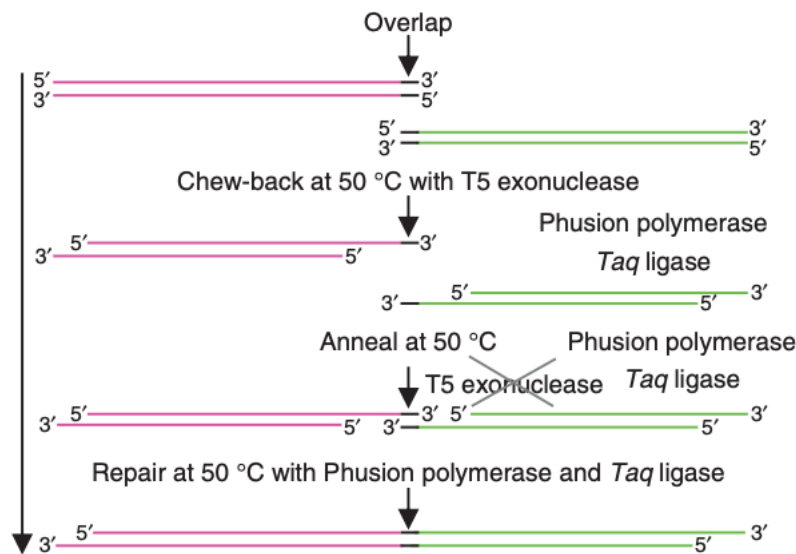
Figure 4. Isothermal assembly reaction. Joining of two adjacent DNA fragments (magenta and green) which share terminal sequence overlaps (in black) in a one-step reaction. Nucleotides on 5' ends are removed by T5 exonuclease, leading to the annealing of the complementary-single stranded DNA overhangs. Gaps are filled by Phusion DNA polymerase and the nicks are sealed by Taq DNA ligase. All the reaction happens at 50ºC, allowing the inactivation of T5 exonuclease.

3.4 MICROBIOLOGICAL TECHNIQUES

3.4.1 Growth conditions and selection media

L. paracasei BL23 was grown in Man, Rogosa and Sharpe (MRS) medium at 37°C without movement or antibiotics. When plated, they were grown in MRS supplemented with agar 2%, and 5µg/ml erythromycin (Em) when indicated.

To store *L. paracasei,* 10 ml overnight culture was centrifuged and resuspended in 500 µl of MRS. 500 µl of Glycerol 50 % were then added and stored at -80°C.

E. coli strains were grown at 37°C in Luria-Bertani broth (LB) media with orbital shaking. When plated, they were grown on LB supplemented with agar 1.5%. Strains D1210 and DC10B were supplemented with 300 µg/ml streptomycin (Sm) and DH5α with 20 µg/ml nalidixic acid (Nx) when indicated. Selective media included the following antibiotics (Apollo Scientific or Sigma Aldrich) at specific concentrations: 100 µg/ml ampicillin (Ap); 25 µg/ml chloramphenicol (Cm); 200 µg/ml Em and 50 µg/ml kanamycin (Km).

To preserve *E. coli* strains, an overnight culture was centrifuged and resuspended in peptone-glycerol (peptone 0.75 % (w/v), glycerol 50% (v/v)) and kept at -20°C and -80°C.

3.4.2 Bacterial conjugation

All matings were performed in solid media. In short, donors and recipients at the stationary phase were mixed in a 1:1 ratio, washed, centrifuged and transferred to a conjugation filter (0.2 µm cellulose acetate filter, Sartorius) on an agar plate. After incubation, the mixture was resuspended on 2 ml of media and several dilutions were performed in order to plate them on selective media.

Transconjugant and donor colonies from the plates were counted and the conjugation frequency was calculated as the number of transconjugant colonies divided by the donors.

Specific strain requirements for conjugation, such as culture growth conditions, incubation time and the media used, are detailed below.

3.4.2.1 From *E. coli* to *E. coli*

Both DH5αT1^R and D1210 were usually used as donors or recipient cells. Both were grown overnight and 100 µl of each culture were used. They were centrifuged and washed in LB. Then, both were mixed in a 1:1 ratio, centrifuged and resuspended on 20 µl of LB. The mixture was placed on a cellulose acetate filter on a LB agar plate and incubated 37°C for 1 hour. After this time, the filter was resuspended on a 2 ml LB tube and several dilutions were performed. Dilutions were plated in LB agar supplemented with the correspondent antibiotics.

3.4.2.2 From *E. coli* to lactobacilli

E. coli strains D1210 or DC10B used as donor cells were grown overnight on LB with the corresponding antibiotics. The recipient *L. paracasei* BL23 was grown overnight on MRS without antibiotics. 100 µl were used from each donors and recipient cultures. They were centrifuged and washed with BHI media (Oxoid). Then, they were both mixed in a 1:1 ratio, centrifuged, and resuspended in 20 µl of BHI. The mixture was placed onto a cellulose acetate filter on a BHI 2% agar plate and incubated at 37°C for 24 hours. After this time, the filter was resuspended on a 2 ml BHI tube and serial dilutions were performed in order to plate on selective media. Donors were plated on LB agar with Sm 300 µg/ml and Ap 100 µg/ml; recipients on MRS agar and transconjugants on MRS agar with Em 5 µg/ml.

3.4.3 *E. coli* electroporation

In order to introduce plasmids into *E. coli* strains by electroporation, electrocompetent cells were prepared as follows: overnight cultures were diluted (1/20), grown to an OD_{600}=0.5-0.7 and centrifuged at 3.500 rpm at 4 during 20 min. Then, cells were washed four times with 1 volume of ice-cold milliQ water, pelleted and the supernatant was discarded. A final wash was performed with 1/50 volume of ice-cold-glycerol 10%. After this step, cells were resuspended in 1/400 volume ice-cold glycerol and aliquoted in 50 µl samples in order to store them at -80°C until usage.

For electroporation, aliquots were mixed with 1-100ng of DNA and transferred into a 0.2 cm Gene Pulser cuvette (BioRad). An electric pulse (2.5 kV/cm, capacitance 25 µF and 200 Ω) was then applied to the cuvette with the mixture in a MicroPulser™ (BioRad). After electroporation, 1 ml of LB was added to the electroporated cells and these were incubated at 37 °C for an hour, to allow antibiotic-resistance gene expression. Then, cells were plated on LB with the appropriate antibiotics for selection of the introduced plasmid.

3.5 Computer analysis

Statistical analyses

For the data analyses and statistical comparisons, the software GraphPad Prism 8.00 (San Diego, CA) was used. For data comparison, student's t-test was used. In the graphs, a significant result is expressed by one or more asterisks.

Software

BLAST. The Basic Local Alignment Search Tool finds regions of local similarity between sequences. It compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. This programme can also be used to infer functional and

evolutionary relationships between sequences as well as help identify members of gene families (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Vector NTI Advance and SnapGene. They are sequence analyses and design tools that may be used to view, build, analyze, transform and share DNA/protein sequences, as well as construct primers for PCR, cloning, sequencing, or hybridization. There is no online version available, but a trial version can be downloaded from their websites. (https://www.thermofisher.com/es/es/home/life-science/cloning/vector-nti-software.html; https://www.snapgene.com).

GraphPad Prism. It is a scientific 2D graphing and statistics software. It is useful for performing different statistical analyses and displaying experimental results in a graphical way. A trial version can be downloaded from their website (http://www.graphpad.com/scientific-software/prism/).

Chromas Lite. It is a DNA sequence viewer, allowing the visualization of sequencing chromatogram files. It is a free software.

4. RESULTS

4.1 Optimization of the conjugation protocol from *E. coli* to lactobacilli

Bacterial conjugation is a mechanism of horizontal DNA transfer from a donor cell to a recipient cell which requires physical contact (Llosa and de la Cruz, 2005). Conjugation from *Escherichia coli* to lactobacilli has been recently described in our lab. For this, a conjugation protocol was developed (section 3.4.2.2) and conjugative systems R388 and RP4 were used to transfer mobilizable shuttle plasmids into the recipient strain. To optimize the conjugation protocol described by (Samperio et al., 2021), several experiments were performed in order to obtain the optimal ratio of donor/recipients and the optimal donor and recipient growth phase. Moreover, to obtain higher conjugation frequencies, a strain deficient in methyltransferase was used as a donor (**Figure 5**).
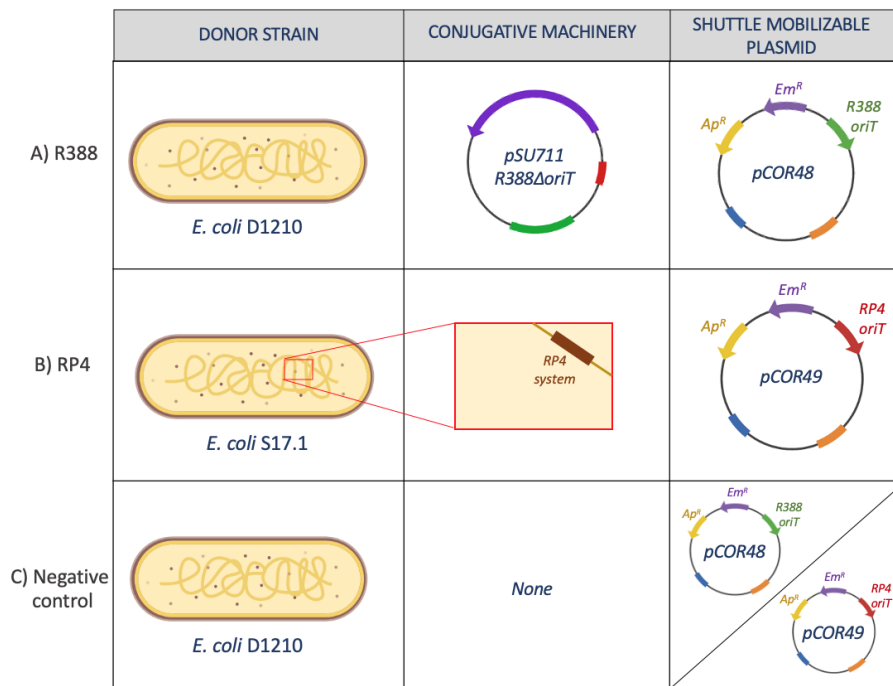

Figure 5. Donor strains with the conjugative machinery and mobilizable plasmids used for conjugation to *L. paracasei*. A) *E. coli* donor strain D1210 with the conjugative system R388 encoded by the plasmid *pSU711* and the shuttle mobilizable plasmid *pCOR48*. B) *E. coli* S17.1 with the conjugative system RP4 integrated on the chromosome and pCOR49. C) The negative control: D1210 without conjugative machinery and the corresponding shuttle mobilizable plasmid.

In this work, R388 and RP4 conjugative systems were used in order to transfer the shuttle mobilizable plasmids pCOR48, carrying the R388 *oriT* and pCOR49, carrying the RP4 *oriT* into the recipient bacteria. *E. coli* D1210 with the helper plasmid pSU711, which codifies the R388 conjugative system, was used as a donor in order to deliver the plasmid pCOR48 (**Figure 5A**). To mobilize the

plasmid pCOR49, the *E. coli* donor strain S17.1 was used, which has the genes codifying the RP4 system integrated in the chromosome (**Figure 5B**). As the recipient strain we used *Lacticaseibacillus paracasei* BL23 (**Table 1**). As a control, conjugations from the *E. coli* strain D1210 with either pCOR49 or pCOR48 and without the helper plasmid providing the rest of the conjugative system were performed (**Figure 5C**). Both shuttle mobilizable plasmids contained resistance genes for ampicillin and erythromycin in order to select the transconjugants.

Conjugation frequencies were calculated as the number of transconjugants divided by the number of donors.

4.1.1 Variation of the physical parameters

In order to obtain higher conjugation frequencies, different parameters of the original protocol can be changed such as incubation time and temperature, ratios between the donors and receptors and bacterial growth phases. Incubation time and temperature were already performed and optimized in our lab, so we focused on the bacterial ratio and growth phases (**Figure 6**).

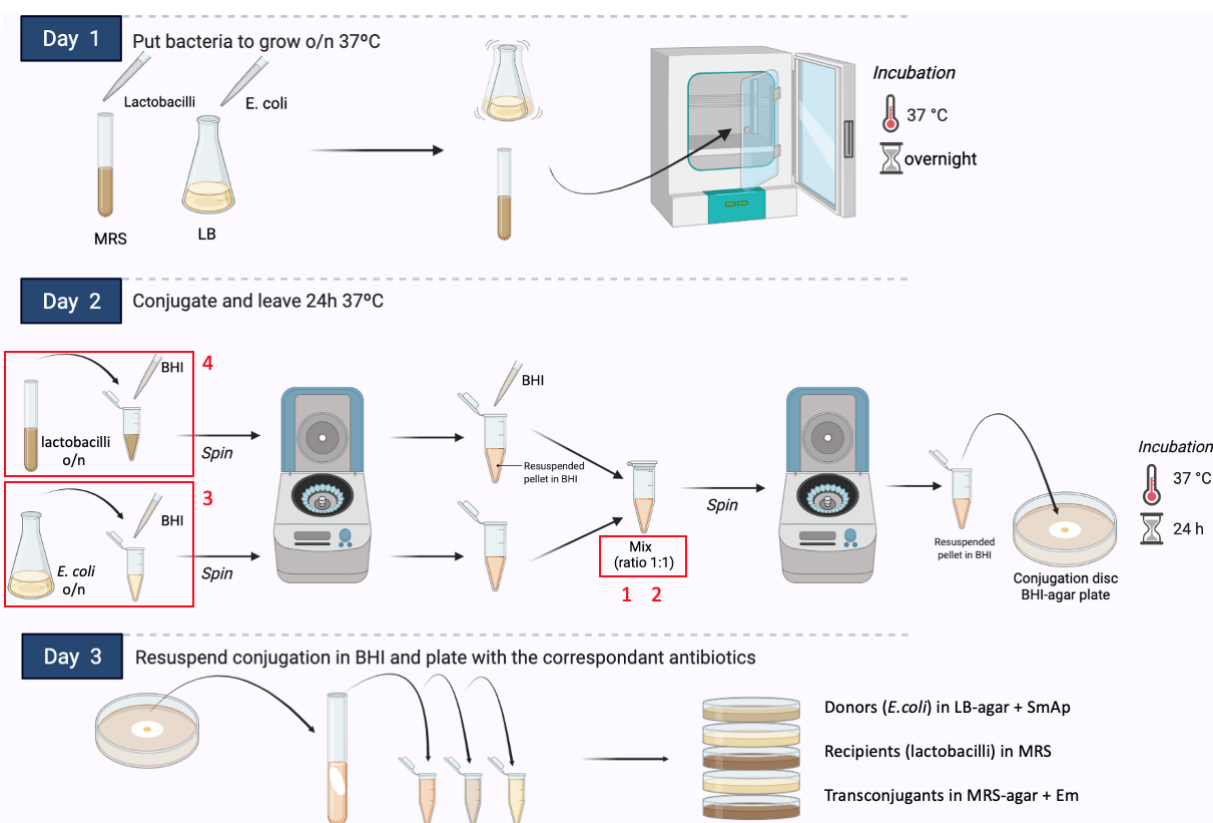**Figure 6**: **Conjugation protocol from *E. coli* to lactobacilli**. Conjugation protocol is described in section 3.4.2.2. Physical parameters modified for this work are marked in red. 1 and 2 correspond to the changes performed for the donors (1) and recipients (2) ratio; 3 correlates with variations in recipient growth phases and 4 with the donor's growth phases. Created with BioRender.com.

4.1.1.1 Variation of bacterial ratio

First, donor's ratio was modified from the normal condition of 1:1 (donors/recipients) ratio to 5:1 and 10:1 with R388 conjugative system. This was performed by leaving a constant volume of 100 µl of recipients and mixing them with 100, 500 and 1000 µl respectively.

As is shown in **Table 4** and **Figure 7**, no significant increase of the conjugation frequency was observed when we modified the donor's ratio with R388. Moreover, when it varied to 10:1 a significant decrease of the conjugation frequency was observed, compared to the normal condition (1:1).

Table 4. Conjugation from _E. coli_ to _L. paracasei_ variating the donor's ratio with the R388 conjugative machinery.

Ratio [1]		Conjugation frequencies [2]	
		R388	
D	R	+	-
1	1	3.46×10^{-7} ($\pm 9.97 \times 10^{-8}$)	$< 2.10 \times 10^{-7}$ ($\pm 2.97 \times 10^{-7}$)
5	1	4.44×10^{-7} ($\pm 4.33 \times 10^{-7}$)	$< 2.05 \times 10^{-7}$ ($\pm 2.55 \times 10^{-7}$)
10	1	3.11×10^{-8} ($\pm 3.54 \times 10^{-10}$)	$< 2.94 \times 10^{-8}$ ($\pm 3.99 \times 10^{-8}$)

Data represent the mean of 2 independent experiments.

Conjugation from _E. coli_ into the _L. paracasei_ BL23 recipient strain was performed.

[1]Ratios of donors (D) were varied. [2]The conjugation system was provided by the helper plasmid pSU711 for R388 (+). _E. coli_ strain D1210 without the conjugation system was used as a negative control (-).
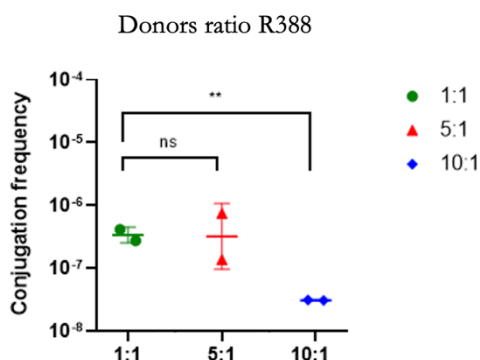

Figure 7. Conjugation frequencies obtained from varying donor's ratio with R388 conjugative system. 1:1 (donors/recipients) ratio was stablished as the normal condition and then donor's ratio was varied from 1:1 to 10:1 using the R388 conjugative system.

Seen the results obtained for the R388 conjugative system, we decided to perform the following conjugations with the RP4 system, as its efficiency is higher. For this, donor's ratio was changed from the normal condition of 1:1 (donors/recipients) ratio to 5:1, 10:1, 100:1 and 1000:1. This was performed by leaving a constant volume of 100 µl of recipients and mixing it with 100, 500 and 1000µl as before. For ratios 100:1 and 1000:1 the recipients volume used was varied, taking 10

and 1 µl respectively (**Table 5 and Figure 8a**). Then, conjugation was performed as the original protocol.

Recipients ratio was varied from the normal condition of 1:1 (donors/recipients) ratio to 1:10 and 1:1000. A constant volume of donors of 10 µl was stablished and then, 10, 100 and 1000 µl of the recipient overnight culture were added respectively. Conjugations were performed only with the RP4 conjugative system (**Table 5 and Figure 8b**).

In **Table 5 and Figure 8** the results obtained from varied bacterial ratio are summarized. No significant increase of the conjugation frequency was observed when we modified either the donors or recipient's ratio with the RP4 conjugative system. Moreover, when we varied the recipient's ratio to 10:1, we observed a significant decrease of the conjugation frequency, compared to the normal condition (1:1) (**Figure 8 b**).

Table 5. Conjugation from *E. coli* to *L. paracasei* variating the ratio with the RP4 conjugative machinery.

Ratio [1]		Conjugation frequencies [2]	
		RP4	
D	**R**	**+**	**-**
1	1	7.24×10^{-5} ($\pm\ 1.12 \times 10^{-4}$)	$< 1.58 \times 10^{-7}$ ($\pm\ 2.22 \times 10^{-7}$)
10	1	1.13×10^{-4} ($\pm\ 1.69 \times 10^{-4}$)	$< 3.62 \times 10^{-7}$ ($\pm\ 2.77 \times 10^{-7}$)
100	1	2.96×10^{-5} ($\pm\ 2.93 \times 10^{-5}$)	$< 1.68 \times 10^{-7}$ ($\pm\ 2.59 \times 10^{-7}$)
1000	1	5.54×10^{-5} ($\pm\ 4.86 \times 10^{-5}$)	$< 1.77 \times 10^{-7}$ ($\pm\ 1.62 \times 10^{-7}$)
D	**R**	**+**	**-**
1	1	5.37×10^{-6} ($\pm\ 3.03 \times 10^{-6}$)	$< 5.97 \times 10^{-7}$ ($\pm\ 9.73 \times 10^{-7}$)
1	10	5.17×10^{-7} ($\pm\ 2.84 \times 10^{-7}$)	$< 1.66 \times 10^{-8}$ ($\pm\ 4.19 \times 10^{-9}$)
1	100	5.95×10^{-6} ($\pm\ 9.05 \times 10^{-6}$)	$< 1.60 \times 10^{-8}$ ($\pm\ 9.50 \times 10^{-9}$)

Data represent the mean of 3 independent experiments.

Conjugation from *E. coli* into the *L. paracasei* BL23 recipient strain was performed.[1]Ratios of either donors (D) or recipients (R) were varied. [2] The conjugation system was provided by the S17.1 chromosome for RP4 (+). *E. coli* strain D1210 without the conjugation system was used as a negative control (-).
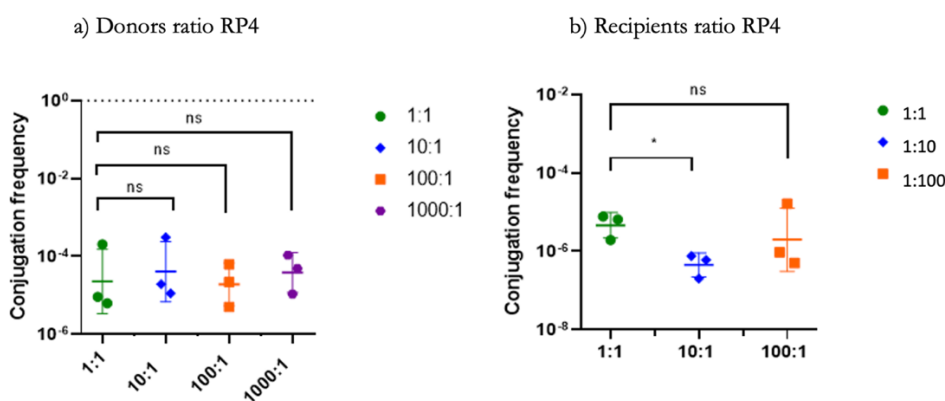

Figure 8. Conjugation frequencies obtained from variating bacterial ratio with RP4 conjugative system. 1:1 (donors/recipients) ratio was stablished as the normal condition. Graph a shows donors' ratio variation from 1:1 to 1000:1 using the RP4 system and variating the receptors volume. Graph b shows the variations from 1:1 to 100:1 performed on the recipients' ratio with the RP4 conjugative system. *, $p < 0.05$; ns, not significant.

4.1.1.2 Variation of bacterial growth phases

In the conjugation protocol, both donor and recipient bacteria in stationary phase from overnight cultures are employed. In order to have the donors in the three different phases: lag (or early exponential), log (exponential) and stationary, three ten-fold serial dilutions were performed from the overnight culture and left to grow at 37°C for two hours. Then, the optical density (OD_{600}) of each culture was measured in order to adjust the same ratio (1:1) donors/recipients. The OD_{600} obtained was around 0.05 for the lag or early exponential, 0.5 for the log and 2 for the stationary phase. Then, the corresponding volume of donors for each of the growing phases was mixed with 100 µl of recipients overnight culture. Conjugations performed with donors overnight culture were established as the normal condition for this experiment. Only the RP4 conjugative system was used.

Recipients in the three growing phases were obtained as the donors. Three ten-fold serial dilutions were performed from the overnight culture and left to grow at 37°C for two hours. Then, the OD_{600} of each sample was measured and compared to the recipients overnight culture in order to have the same donors/receptors ratio. Conjugations performed with the recipients overnight cultures were established as the normal condition. All conjugations were performed with the RP4 conjugative system. Results are summarized in **Table 6 and Figure 9.**

Our hypothesis was that performing conjugation with bacteria on a growth phase different than stationary would allow us to increase the conjugation frequency. However, no significant increase of the conjugation frequency was observed for either donors or recipients on log and lag growing phases. If any, we observed lower conjugation frequencies when the donor strain was not in stationary phase, although the differences were not statistically significative.

Table 6. Conjugation from *E. coli* to *L. paracasei* with bacteria on different growing phases.

Growth phase[1]		Conjugation frequencies (RP4) [2]	
D	**R**	**+**	**-**
Sta.	Sta.	5.44×10^{-6} ($\pm 8.32 \times 10^{-6}$)	$< 5.08 \times 10^{-8}$ ($\pm 6.46 \times 10^{-8}$)
log	Sta.	3.09×10^{-6} ($\pm 2.99 \times 10^{-6}$)	$< 3.87 \times 10^{-6}$ ($\pm 6.27 \times 10^{-6}$)
lag	Sta.	4.10×10^{-7} ($\pm 4.39 \times 10^{-7}$)	$< 5.56 \times 10^{-4}$ ($\pm 9.62 \times 10^{-4}$)
D	**R**	**+**	**-**
Sta.	Sta.	6.46×10^{-5} ($\pm 7.08 \times 10^{-5}$)	$< 2.59 \times 10^{-7}$ ($\pm 2.26 \times 10^{-7}$)
Sta.	log	1.09×10^{-5} ($\pm 8.93 \times 10^{-6}$)	$< 2.85 \times 10^{-7}$ ($\pm 3.13 \times 10^{-7}$)
Sta.	lag	2.88×10^{-5} ($\pm 2.92 \times 10^{-5}$)	$< 2.71 \times 10^{-7}$ ($\pm 2.12 \times 10^{-7}$)

[1] Growth phases from donors (D) and recipients (R) were varied from stationary (Sta) to exponential (log) and lag. [2]The conjugation system RP4 was provided by the S17.1 chromosome. Conjugation was performed from *E. coli* into *L. paracasei* BL23 recipient strain. Conjugation frequencies were calculated from variating the growth phase of both donors and recipients (+), D1210 without the conjugation system was used as a negative control (-). Data represent the mean of at least 3 independent experiments.
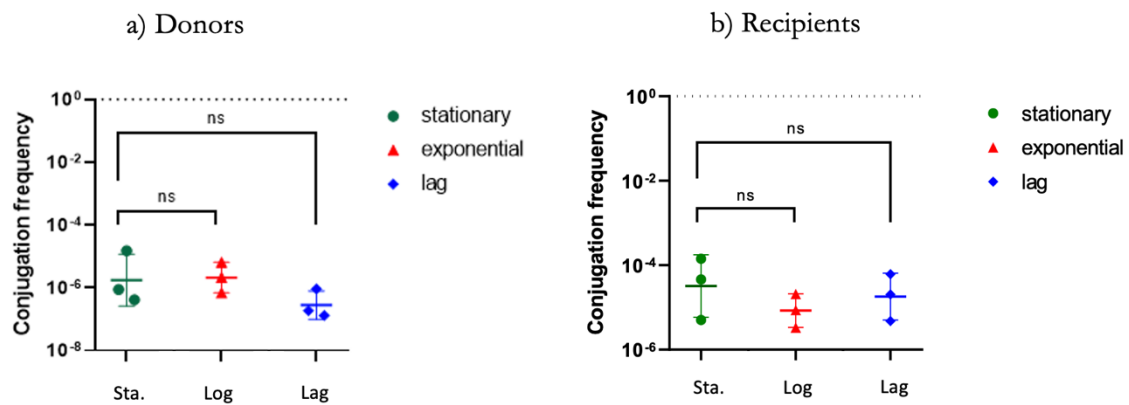
Figure 9. Conjugation frequencies from different bacterial growth phases. Stationary phase from overnight culture was established as the normal condition and conjugation frequencies were obtain from exponential and lag phases of a) donors or b) recipients. The conjugative system used was RP4. Ns; not significant.

4.1.2 Conjugative transfer of unmethylated DNA

Bacteria have developed the ability to recognize and distinguish self-DNA from foreign DNA. In order to restrict the constant exposure to external DNA different strategies have been developed. Restriction-modification (RM) systems are defence barriers inside the bacteria which specifically recognize the methylation status of incoming DNA (Vasu and Nagaraja, 2013). Therefore, foreign DNA with a different methylation pattern is recognized as non-self and cleaved. For this reason, they have been described to be partly responsible of the low transformation efficiencies obtained in wild type strains. In order to bypass this barrier, several studies performed successful transformations by introducing non-methylated DNA in lactobacilli (Spath et al., 2012) and also, using an *E.coli* strain deficient in Dcm methyltransferase as a conjugative donor in *Staphylococcus* (Monk et al., 2012). In this work we performed conjugations to *Lacticaseibacillus paracasei* BL23 with the *E. coli* strain DC10B (**Table 1**), deficient in methyltransferase (dcm⁻), expecting to see an increase in the conjugation frequencies obtained.

The dcm⁻ strain with the R388 and RP4 conjugative systems was used as a donor. In order to prepare the dcm⁻ strain, the pSU2007 plasmid, which encodes the R388 system, was introduced by conjugation as well as pRL443, which encodes the RP4 conjugative system into the other dcm⁻ donor strain, and both pCOR48 and pCOR49 mobilizable plasmids were introduced by electroporation respectively. For comparison with methylation-proficient donor strains, we used the D1210 strain with the pSU2007 helper plasmids for the R388 system. For the RP4 system we used D1210 with pRL443 helper plasmid. All with the corresponding mobilizable plasmids for each conjugative system. For the negative control we used the dcm⁻ mutant and the D1210 strain with just the mobilizable plasmids, and not the conjugative plasmid (**Figure 10**).
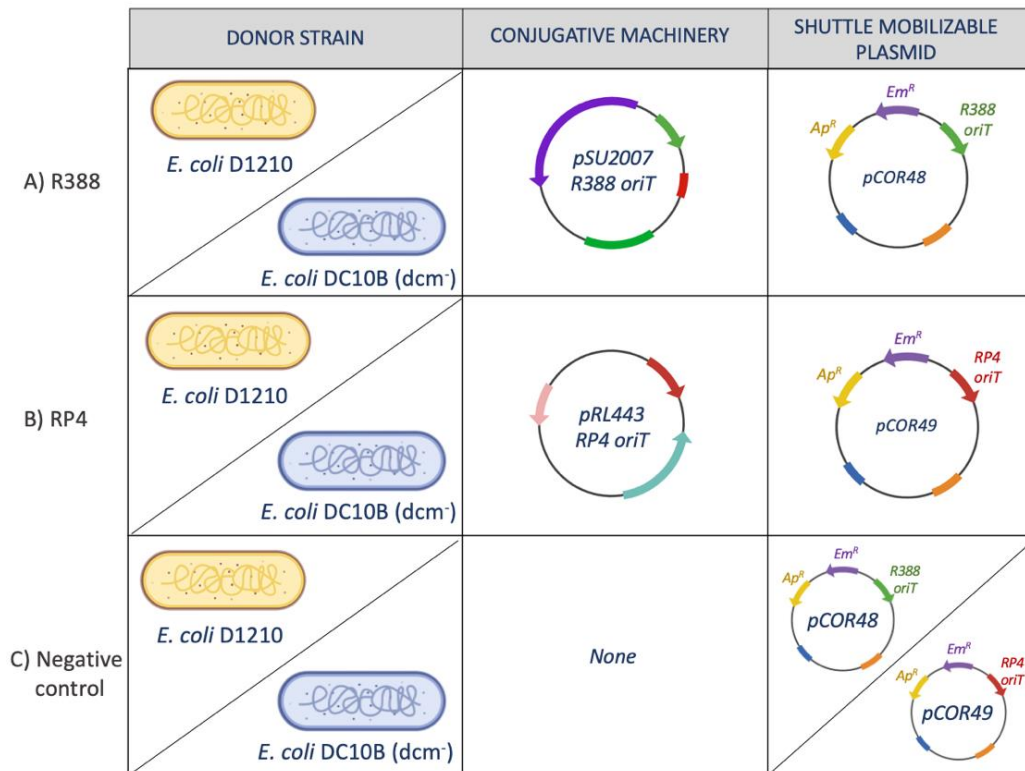
Figure 10. Donor strains with RP4 or R388 for conjugation to *L. paracasei*. A) R388 conjugative system. *E. coli* donor strain D1210 and dcm⁻ DC10B with the helper plasmid *pSU2007* and the shuttle mobilizable plasmid *pCOR48*. B) RP4 conjugative system. *E. coli* D1210 and dcm⁻ DC10B with the helper plasmid pRL443 and pCOR49. C) The negative control: D1210 and dcm⁻ DC10B without conjugative machinery and the correspondent shuttle mobilizable plasmid.

So far, the helper plasmid used for R388 conjugative system was pSU711. This plasmid does not have an *oriT* for R388 and hence it just mobilizes the shuttle plasmid but not itself. In this case, in order to prepare the donor dcm⁻ strain DC10B, a plasmid with an *oriT* for R388 which could transfer itself via conjugation was needed. For this reason, helper plasmid pSU2007 was used instead pSU711. So, first we wanted to prove that there was not any difference between the mobilization frequency of both helper plasmids. Therefore, we performed several conjugations from a D1210 donor strain with either the pSU711 or the pSU2007 helper plasmids into DH5α and *L. paracasei* BL23 (**Table 7**). Results showed that no significant difference of the conjugation frequency was obtained between the efficiency of both helper plasmids to *E. coli* (**Figure 11a**) or *L. paracasei* BL23 (**Figure 11b**) as expected, so we continued with the experiment.
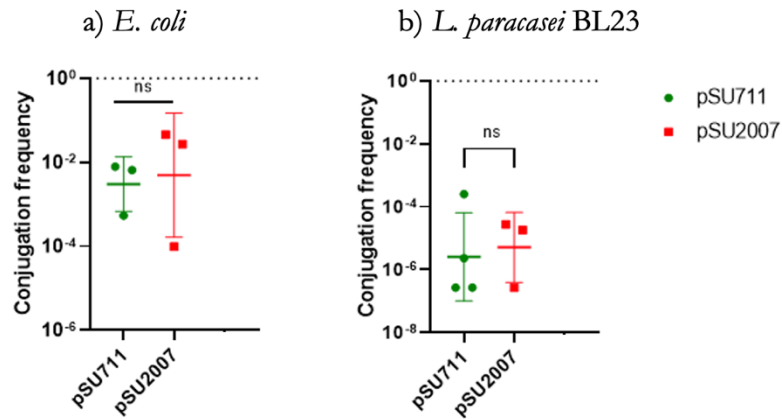
Figure 11. Conjugation frequencies using R388 pSU711 or pSU2007 as helper plasmids. Conjugation performed from *E. coli* strain D1210 with helper plasmid pSU711 or pSU2007 into a) *E. coli* strain DH5a or b) *L. paracasei* BL23. Donors carried the mobilizable plasmid pCOR48.

Table 7 and **Figure 12** summarize the results of the conjugation from the dcm⁻ strain with the R388 and RP4 conjugative systems into *L. paracasei* BL23. Unfortunately, conjugation frequency was not significantly increased when we transferred DNA deficient in methylation with the R388 system, compared to D1210. Moreover, when we used the mutant strain with the RP4 conjugative system, no transconjugants were reported in any of the replicates performed. However, it has to be noted that the conjugation frequency from D1210 was close to the detection limit when the RP4 derivative pRL443 was used as a helper plasmid. Since this low frequency was unexpected for a RP4-mediated mobilization, we compared the efficiency as providers of the RP4 transfer system of the pRL443 helper plasmid with the strain S17.1 that we were using previously. For this, we performed conjugations from either D1210 with the pRL443 helper plasmid or S17.1 into *L. paracasei* BL23 (**Figure 13**). The result shows that conjugation frequency is significantly higher for S17.1 (more than two logs), obtaining conjugation frequencies with the pRL443 helper plasmid close to the detection limit of the assay. This explains the lack of transconjugants observed when using the Dcm mutant strain as donor carrying the same plasmids (**Table 7**).

Table 7. Conjugation from strain deficient in Dcm methyltransferase to *L. paracasei* and *E. coli* using R388 and RP4 conjugative systems

Recipient	Donor (*E. coli*)	Dcm methylase	Conjugation system[1]	Mobilizable plasmid	Conjugation frequency
L. paracasei [2]	D1210 (pSU2007)	+	R388	pCOR48	**1.14×10^{-5} ($\pm 1.35 \times 10^{-5}$)**
	D1210 (pSU711)	+	R388	pCOR48	**6.40×10^{-5} ($\pm 1.26 \times 10^{-4}$)**
	D1210	+	none	pCOR48	$< 5.59 \times 10^{-7}$ ($\pm 4.94 \times 10^{-7}$)
	DC10B dcm⁻ (pSU2007)	-	R388	pCOR48	**4.50×10^{-6} ($\pm 8.90 \times 10^{-6}$)**
	dcm⁻	-	none	pCOR48	$< 7.27 \times 10^{-8}$ ($\pm 2.59 \times 10^{-8}$)
	D1210 (pRL443)	+	RP4	pCOR49	**4.67×10^{-8} ($\pm 3.10 \times 10^{-8}$)**
	S17.1	+	RP4	pCOR49	**1.61×10^{-5} ($\pm 1.91 \times 10^{-5}$)**
	D1210	+	none	pCOR49	$< 1.39 \times 10^{-8}$ ($\pm 3.75 \times 10^{-9}$)
	DC10B dcm⁻ (pRL443)	-	RP4	pCOR49	**$< 9.85 \times 10^{-9}$ ($\pm 1.16 \times 10^{-8}$)**
	dcm⁻	-	none	pCOR49	$< 9.82 \times 10^{-9}$ ($\pm 8.14 \times 10^{-10}$)
E. coli [3]	D1210 (pSU2007)	+	R388	pCOR48	**2.44×10^{-2} ($\pm 2.31 \times 10^{-2}$)**
	D1210 (pSU711)	+	R388	pCOR48	**4.99×10^{-3} ($\pm 3.91 \times 10^{-3}$)**
	D1210	+	none	pCOR48	$< 5.12 \times 10^{-7}$ ($\pm 7.43 \times 10^{-}$)

[1]The conjugation system was provided by the helper plasmids pSU2007 and pSU711 for R388, or by the pRL443 and the S17.1 chromosome for RP4. [2, 3] Conjugation into the *L. paracasei* BL23 and *E. coli* DH5☐ recipient strains. Data represent the mean of at least 3 independent experiments.
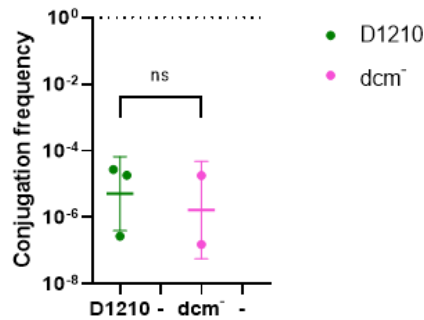

Figure 12. Effect of Dcm methylation on conjugation frequencies from *E. coli* to *L. paracasei*. Conjugation performed from donors *E. coli* D1210 or the mutant strain (dcm⁻) into *L. paracasei* BL23. Both donors carried the helper plasmid pSU2007 with the conjugative system R388 and the mobilizable plasmid pCOR48.
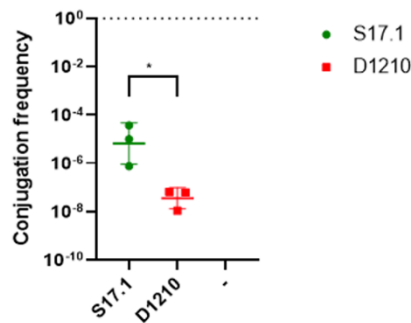

Figure 13. Conjugation frequencies using different sources of the RP4 conjugative system. Conjugation performed from *E. coli* strain D1210 with helper plasmid pRL443 or S17.1 with the RP4 system in the chromosome into *L. paracasei* BL23. Both donors carried the mobilizable plasmid pCOR49.

4.2 Design of a genetic modification tool in lactobacilli introduced by conjugation

4.2.1 Overview of the genetic modification process

Our purpose was to introduce homologous recombination cassettes in lactobacilli by bacterial conjugation. As a proof of concept, we aimed to insert an erythromycin resistance cassette in the place of the *lacG* gene in lactobacilli's genome. This cassette would be composed by two sequences homologous to the ones adjacent to *lacG* in the genome flanking the antibiotic resistance gene. By cloning the HR cassette in mobilizable plasmids, it can be introduced in the target strain by conjugation. A simple approach which will allow us to perform homologous recombination on lactobacilli strains which cannot be transformed with the plasmid DNA.

The cassette was going to be cloned in two different vectors: pCOR51 and pEM::*oriT*$_{RP4}$. The first one is derived from pUC8 with an *oriT* for RP4. It only has an *oriV* for *E. coli*, being a suicide plasmid for lactobacilli. pEM::*oriT*$_{RP4}$ is a shuttle vector derived from *pEM94* with an *oriV* for *E.coli* and an *oriV* thermosensitive isolated from *Lactococcus lactis cremoris* (Martín et al., 2004). The *oriT* for RP4 was inserted into this plasmid. This will allow us to introduce both vectors with the cassette into the bacteria by conjugation, making sure that there will be no further copies of any of them once the recombination has happened; pCOR51 will not replicate, and pEM::*oriT*$_{RP4}$ can be maintained in the recipient for as long as we want, in order to increase the chance of homologous recombination to happen, but then the episomal plasmid can be removed by shifting the temperature. In order to analyse if the recombination occurred, since *lacG* is replaced by the erythromycin gene, lactobacilli will be plated on MRS with Em and on X-gal, obtaining a white colony phenotype instead of blue, as the *lacG* will not be functional. The homologous recombination plasmid and its design are described in **Figure 14.**

4.2.2 Design and construction of a suicide mobilizable plasmid carrying a recombination cassette.

Plasmids constructed for this work are resumed in **Table 8**.

Table 8. Plasmid constructions designed for this work

Plasmid	Description	Phenotype	Construction [1]		
			Vector	Insert	Oligonucleotides [2]
pCOR51::*em+* HR *lacG*	pCOR51: *em*^{R}: *lacG* homologous recombination cassette	Em^R	pCOR51	*lacG$_1$* *em* *lacG$_2$*	**Insert$_1$**: Tail pCOR51 + *lacG$_1$* F; *lacG$_1$* R **Insert$_2$**: Tail *lacG$_1$*+ *em* R; Tail *lacG$_2$*+ *em* F **Insert$_3$**: *lacG$_2$* F; Tail pCOR51 + *lacG$_2$* R **Vector**: pCOR51 F; pCOR51 R
pEM::*oriT*$_{RP4}$:: *em+*HR *lacG*	pEM::*oriT*$_{RP4}$: *em*^{R}: *lacG* homologous recombination cassette	Cm^R, Em^R	pEM::*oriT*$_{RP4}$	*lacG$_1$* *em* *lacG$_2$*	**Insert$_1$**: Tail pEM::*oriT* + *lacG$_1$* F; *lacG$_1$* R **Insert$_2$**: Tail *lacG$_1$*+ *em* R; Tail *lacG$_2$*+ *em* F **Insert$_3$**: *lacG$_2$* F; Tail pEM::*oriT* + *lacG$_2$* R **Vector**: pEM::*oriT* F; pEM::*oriT* R

[1]Design for isothermal assembly reactions. [2]Oligonucleotides for the amplification of the insert or the vector are mentioned; their sequence is described in **Table 3.**

In order to design the plasmid with the recombination cassette, three inserts were amplified separately to be cloned into the vectors by isothermal assembly. The erythromycin resistance gene, *em* (958 pb) was amplified from the pCOR49 plasmid using the appropriate primers: *em F* and *em R* (**Table 3**). The other two inserts were amplified from the genome of *L paracasei* BL23 (GeneBank: FM177140.1) as they are the two sequences 5' and 3' to the *lacG* gene. We will refer to these as *lacG₁* (826 bp) and *lacG₂* (756 bp). For their amplification we used the primers: *lacG₁* F; *lacG₁* R; *lacG₂* F; *lacG₂* R (**Table 3**). The two vectors were linearized by the amplification of the pCOR51 plasmid without the erythromycin resistance gene (pCOR51 F and pCOR51 R), and the pEM::*oriT*$_{RP4}$ without the resolvase (pEM::*oriT*$_{RP4}$ F and pEM::*oriT*$_{RP4}$ R). For the assembly, *em* F has a 30 bp 3' tail complementary to the 3' end of *lacG₁* R, and *em* R has a 30 bp 3' tail complementary to the 3' end of *lacG₂* F. The *lacG1* F primers carried 30 bp 3' ends complementary to each vector's R primers, and the *lacG₂* R primers, complementary to the vectors F primers. The isothermal assembly reaction of the three inserts with the two vectors is summarized in **Figure 14.**
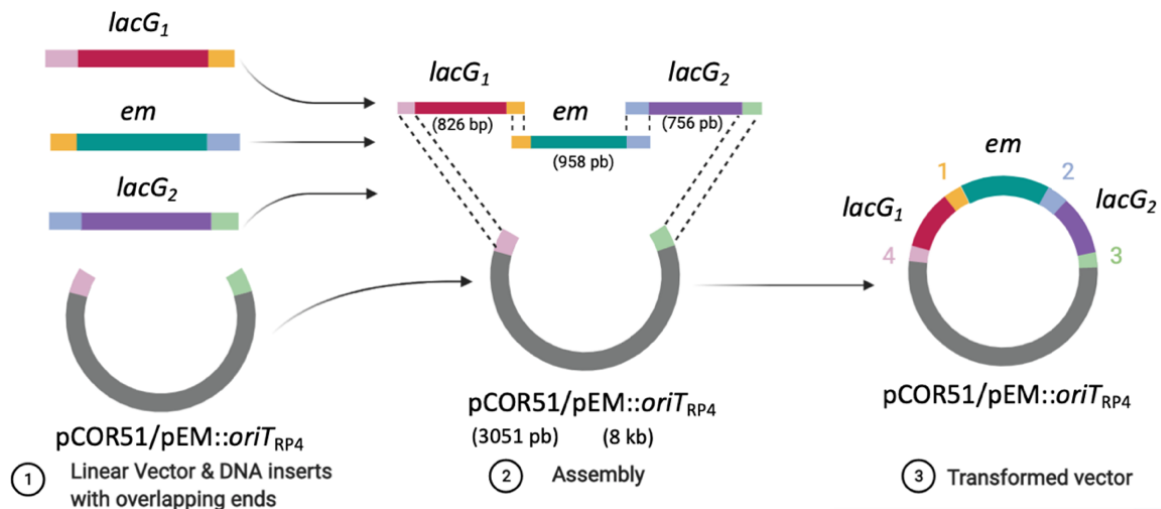

Figure 14. Design of the construction of a mobilizable plasmid containing the homologous recombination cassette. Three inserts: *lacG₁*, erythromycin resistance gene (*em*) and *lacG₂* are assembled and inserted in two different vectors: pCOR51 and pEM::*oriT*$_{RP4}$. Assembly 1 corresponds to *lacG₁* and *em*; 2 to *em* and *lacG₂*; 3 to *lacG₂* and the vector and 4 to the vector and *lacG₁*. Created in BioRender.com.

In order to see if the inserts and vector fragments were the size we expected, we visualized them on an agarose gel. As can be seen in **Figure 15**, all the sizes obtained were as predicted. PCR products were treated with DpnI in order to remove the plasmid templates and continue with the isothermal assembly reaction.
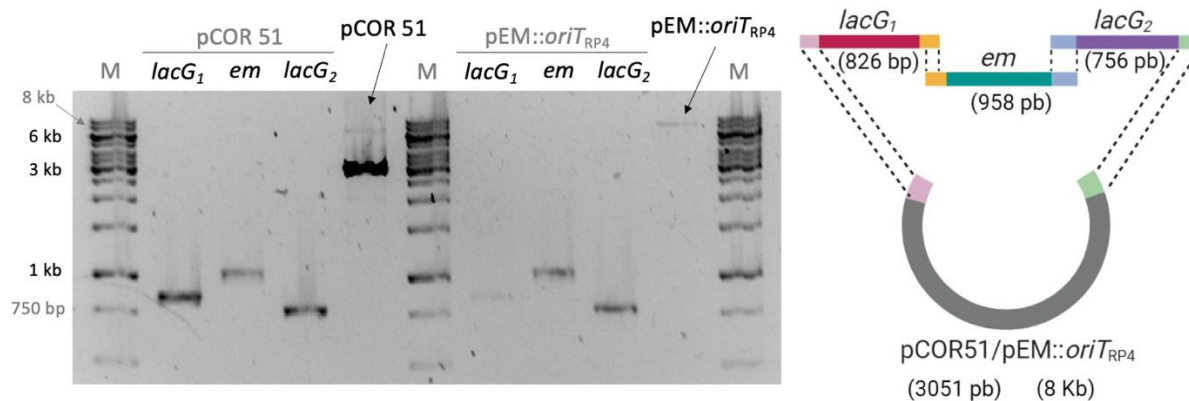
Figure 15. Size of the amplicons for Isothermal assembly. On the left, the agarose gel with the inserts and vectors amplified. Line M, GeneRuler 1kb DNA ladder (Thermo Scientific). On top of the inserts it is detailed in grey the vector they will assemble to, pCOR51 or pEM::*oriT*RP4. Then both vectors were also analyzed. On the right, an explicative image of the inserts, vectors and their corresponding sizes. Created in BioRender.com.

Isothermal assembly reaction was performed for each vector in a ratio 1:1:1:1, 1:2:2:2 and 1:3:3:3 (vector/inserts) as described in section 3.3.6. For the negative control, the same ratios were mixed on water, instead of the isothermal mix, and then the same steps of the reaction were performed. We electroporated the reaction product into the *E. coli* strain DH5α (section 3.4.3). Then, we put to grow the transformed bacteria on LB with erythromycin 200 µg/ml. 30 colonies were grown for the plasmid pCOR51::em+HR *lacG* for the 1:1:1:1 ratio. For the negative control of this plasmid, two colonies were grown. For the other plasmid, pEM::*oriT*RP4::em+HR *lacG*, 7 colonies were reported for the 1:2:2:2 ratio and 2 for the 1:1:1:1. No colonies were grown in the corresponding negative control. All colonies obtained were analyzed by a colony PCR to amplify junctions 1 and 2 (primers *lacG₁* F and *lacG₂* R with the corresponding tails) (**Table 3**). The gel obtained from this PCR was empty, including the positive control of genomic DNA from *L. paracasei* BL23, which should amplify a fragment of 3026 bp containing *lacG*. Since the colony PCR did not seem to work, we performed a PCR directly from the isothermal reaction product to amplify the junction 1 (primers *lacG₁* F and *em* R). Then, we measured the size in an agarose gel and the length obtained was the expected (**Figure 16**), implying that the isothermal assembly was working.

In order to ensure that the isothermal product was correctly assembled, we extracted the plasmid DNA from three of the transformants obtained for each plasmid and performed a restriction analysis with SalI. As it can be seen in **Figure 17**, the restriction pattern obtained did not correspond to the expected products. In the case of colonies obtained from the isothermal assembly on pEM:oriT_{RP4}, the pattern is identical to pCOR49, the template plasmid for the *em* insert amplification. Thus, indicating that in spite of the DpnI treatment, some contamination of the template DNA remained, rendering EmR transformants. In the case of the colonies obtained upon transformation of the isothermal assembly on pCOR51, we do not know what the restriction pattern accounts for, but it does not reflect the correct construct.
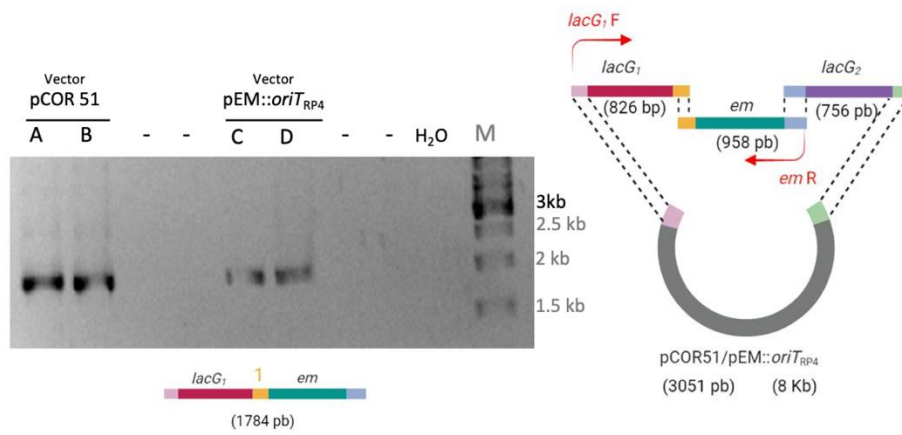
Figure 16. Analysis of the assembly point 1 amplified from isothermal product. Image in the right shows the fragment to be amplified. Arrows in red indicate the direction and name of the primers used. The size expected is specified below the gel, 1784 pb. The agarose gel, in the left, shows the result of the PCR reactions on the isothermal assembly on vector pCOR51 (A, B), vector pEM::*oriT*RP4 (C,D), or the negative control of the isothermal assembly reaction (-). H₂O, control PCR reaction with no template DNA. Line M, GeneRuler 1kb DNA ladder (Thermo Scientific). Created in BioRender.com.
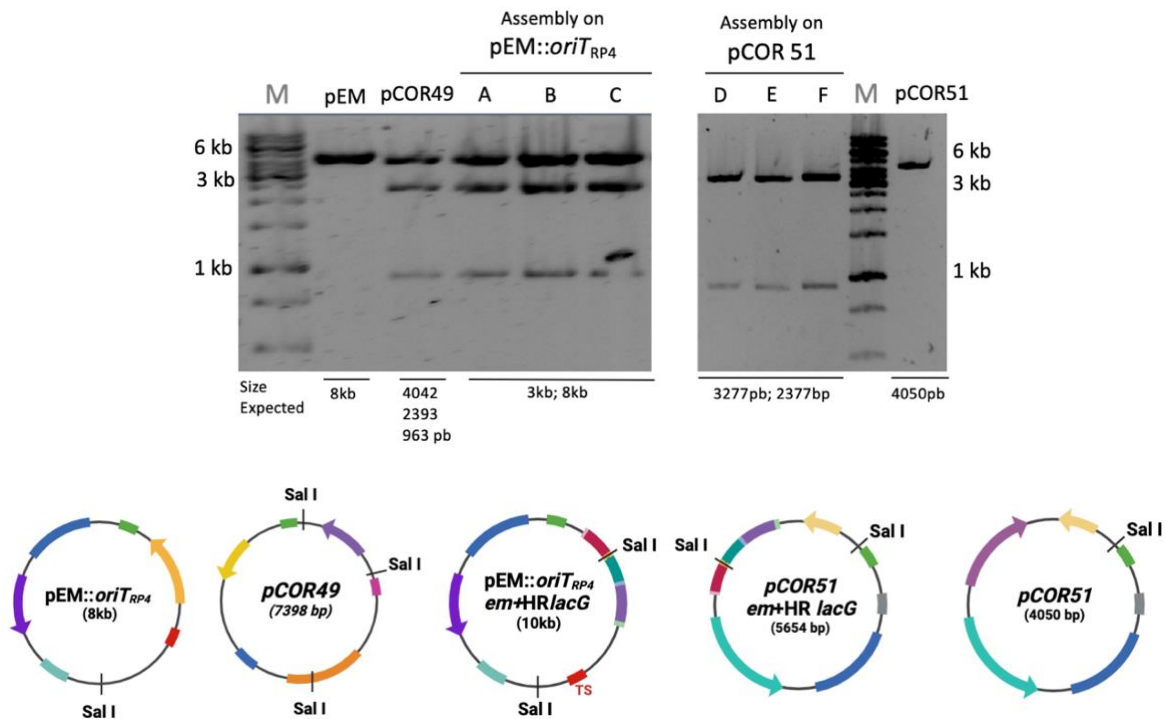

Figure 17. Restriction analysis with SalI. From left to right: M, GeneRuler 1kb DNA ladder (Thermo Scientific); pEM, plasmid pEM::*oriT*RP4 as a control; pCOR49, control; A, B, C three selected colonies transformed with the isothermal product containing vector pEM::*oriT*RP4. On the right: D, E and F correspond to colonies transformed with pCOR51 as the vector; pCOR51, as a control. The expected size of each digestion is detailed in the bottom of the gel. Size obtained from the colonies analyses was not the expected. In D, E and F it matches pCOR49 digestion. Below each plasmid with the Sal I restriction sites.

To check the assembly of the three insert fragments into the vectors in the isothermal assembly reaction product, we amplified the joining regions between the inserts using primers **(Table 3)**: 51F, 51R for the assembly on pCOR51, and RP4F and RP4R for the assembly on pEM::oriT$_{RP4}$. As positive controls for the PCR reactions, we used DNA from both vectors, where these sets of primers should amplify the regions including the *em* gene of pCOR51 (982 bp) and the resolvase gene of pEM::oriT$_{RP4}$ (643 bp). The results are shown in **Figure 18.** We did not obtain any amplification from the isothermal products, and in both positive controls for each vector we obtained the expected size, meaning that primers were working but the assembly was not. As a side note, we also observed some contamination of the vector pCOR51 in the isothermal assembly reaction.

We knew from previous results (**Fig 16**) that at least one of the junctions was being correctly assembled, so the problem was not the assembly reaction itself; thus, at least one of the junctions was not being assembled for other reasons.
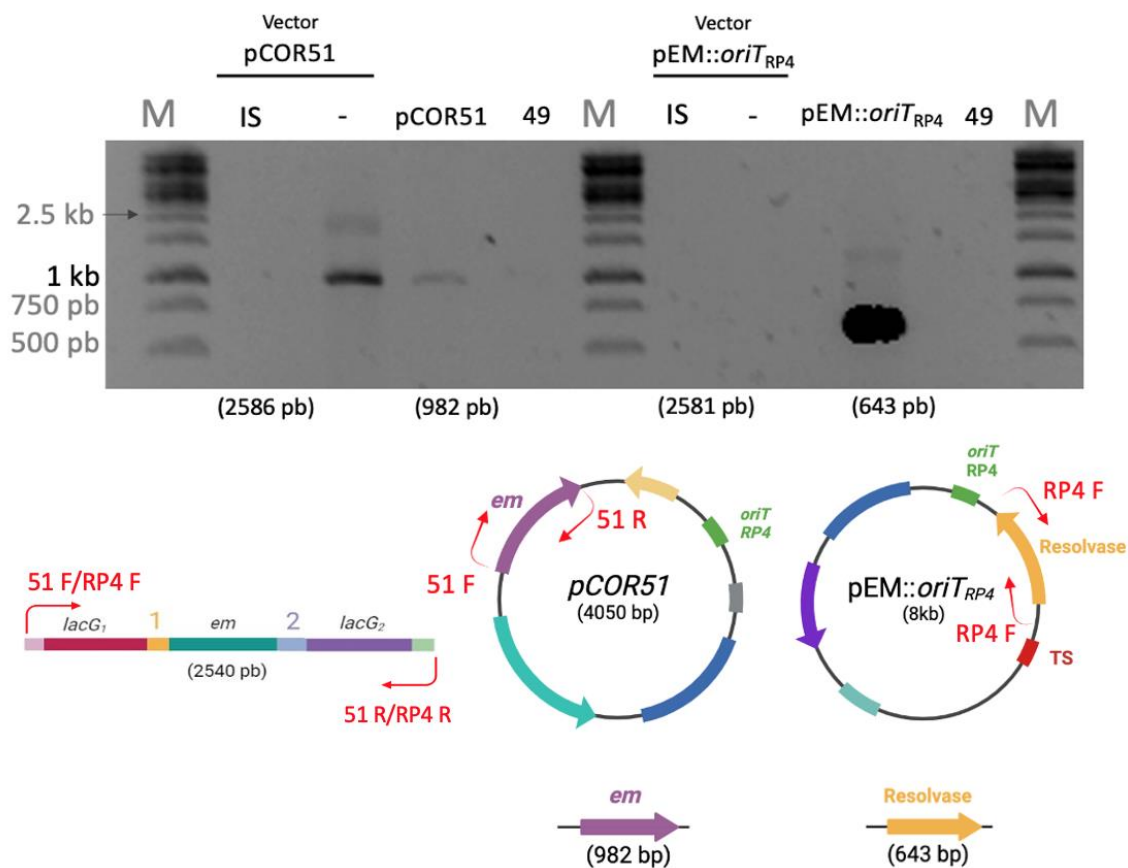

Figure 18. Amplification of the fragment junctions from the isothermal assembly reaction product. On the left, PCR amplification of the three fragments in the assembly with the pCOR51 vector. On the right, the same for pEM::*oriT*$_{RP4}$. From left to right: Lane M, GeneRuler 1kb DNA ladder (Thermo Scientific). IS, amplification of the isothermal product for the correspondent vector. IS-, the negative control of the isothermal assembly. 49, plasmid pCOR49. pCOR51, pCOR49 and pEM::*oriT*$_{RP4}$ were used as controls. Expected size of the amplification is shown on the bottom with the correspondent primers: 51 F, 51 R or RP4 F, RP4 R.

To check which of the junctions were not assembling correctly, we performed four different isothermal reactions in a 1:1 ratio, two for the assembly point 1 and two for the assembly point 2, for each of the different vectors. Then, we amplified the expected assembled product by PCR directly from the isothermal reactions. As it can be seen in **Figure 19**, the assembly number 2 for both vectors, which corresponds with *em* and *lacG₂* joining, did not show the expected size (1714 pb), but rather a fragment of about 1 kb. Assembly number 1 for vector pEM::*oriT*ᵣₚ₄ corresponding to *lacG₁* and *em* did show the size expected (1784 pb). For this reason, we deduct that the assembly of the erythromycin with the *lacG2* fragment was not occurring as expected.
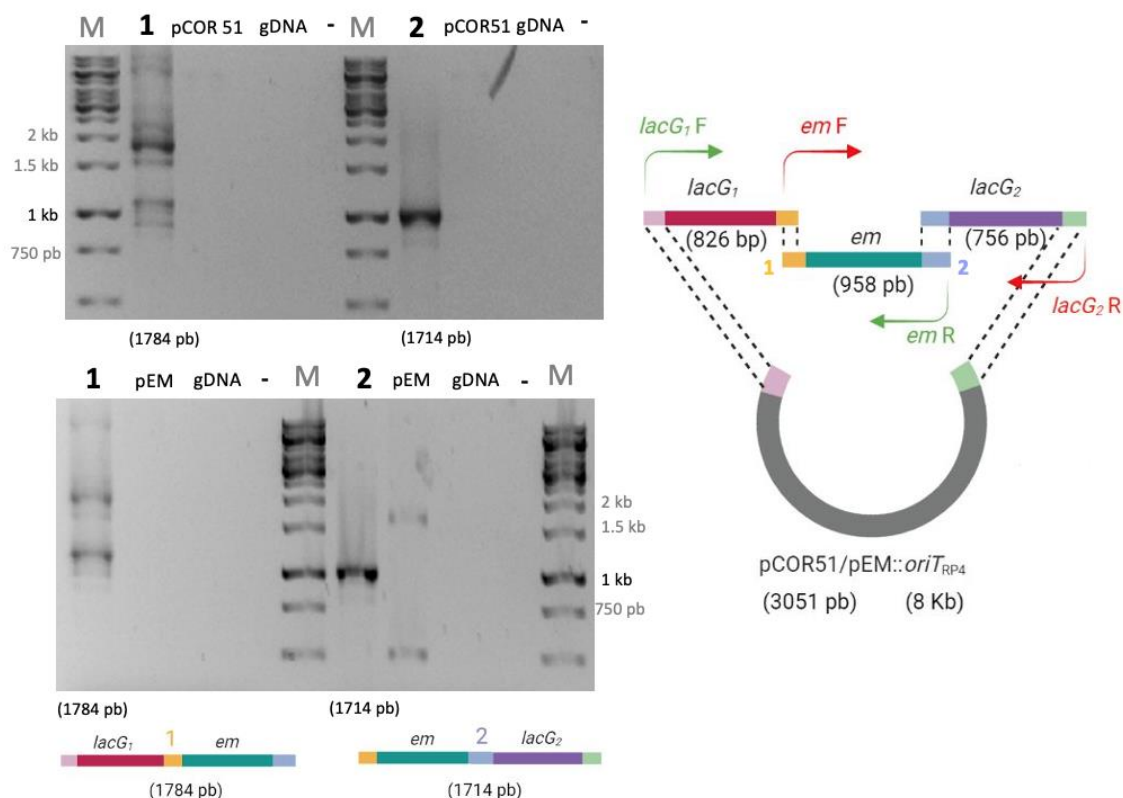

Figure 19. Amplification of the isothermal assembly products from each assembly point. The top corresponds to the isothermal products from the vector pCOR51, below the vector pEM::*oriT*ᵣₚ₄. 1 and 2 refers to the amplification of each assembly point, as described in the figure on the right. pCOR51, plasmid pCOR51. pEM, plasmid pEM::*oriT*ᵣₚ₄. gDNA, genomic DNA from *L. paracasei BL23*. Under the gels, the expected size of each fragment. Lane M, GeneRuler 1kb DNA ladder (Thermo Scientific).

In order to find out why the assembly 2 (*em* with *lacG₂*) was not working, PCR products *em* and *lacG₂* were sent to sequence with primers *em*_F for erythromycin and *lacG₂* F and *lacG₂* R for *lacG₂* (**Table 3**). The result showed the *lacG₂* sequence was as expected, but not the *em* sequence. The sequence of the *em* gene was present, but it did not end as expected (**Figure 20**). It revealed that the 30 bp homology sequence with the *lacG₂* corresponding to the tail of the *em* R oligo was not present. However, 82 additional nucleotides from the pCOR49 were present instead. A detailed analysis of the additional nucleotides made us realise that the end of this pCOR49 extra sequence was extremely

similar to the primer used in forward Tail *lacG₁*+ *em* F, with only two mismatches, leaving a perfect homology of 12 bp in the 3' end. Therefore, the likely explanation (illustrated in **Figure 21**) is that the *em* fragment was amplified on both sides by the same primer, *em* F, thus preventing the correct assembly of the four PCR fragments. This explanation is corroborated by the appearance of the 1 kb band when assembly 2 was checked in Figure 19, corresponding to the *em* gene amplified by primer *em*-F from both ends.
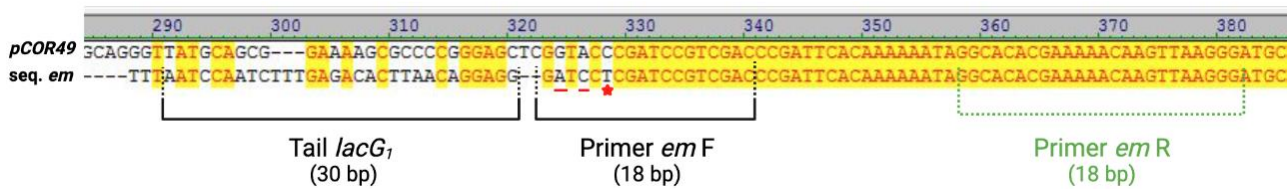

Figure 20. Alignment of pCOR49 and the sequenced *em* PCR fragment. Result of the alignment done with Vector NTI of pCOR49 (top sequence) with the *em* fragment sequenced (lower sequence). The matching nucleotides are marked in yellow. The DNA sequence of primer *em* F and the tail for the assembly to *lacG₁* are indicated in black. The two mismatches of the primer are marked with a red line, and the red asterisk is marking another mismatch which appeared spontaneously (T instead of C). In green, the expected primer *em* R annealing site, designed for the correct amplification of the fragment.
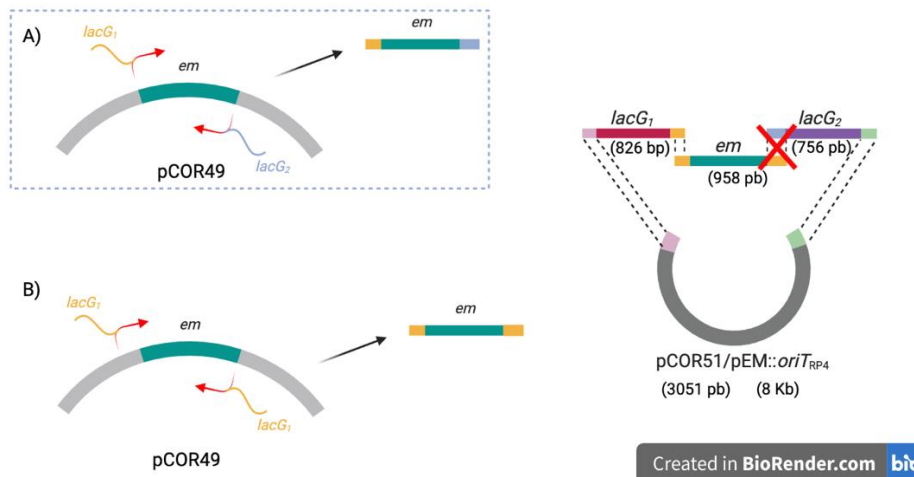
Figure 21. Amplification of the *em* fragment by the same primer. A and B show the PCR amplification of the *em* fragment with the primers marked in red. In A) the *em* amplification expected with the homologous sequences attached to the primers, for *lacG₁* in yellow and for *lacG₂* in blue. B) shows the observed *em* fragment amplification with the same primer in forward and reverse, and therefore only the sequence of homology for *lacG₁*. This explains the reason why the *em* fragment was not assembling to the *lacG₂* fragment, leading into an incomplete plasmid (illustrated in the right part of the Figure).

5. DISCUSSION

Genetic improvement of bacteria is generally achieved by targeted genetic modification and for this, the first step is the introduction of DNA. To date, electroporation has been the most used method for the introduction of genetic material in lactobacilli. However, this group is generally difficult to transform, as electroporation frequencies are usually low and strain-dependent, especially for some wildtype strains for which sometimes transformation is not even feasible. Therefore, in order to transfer DNA into lactobacilli, other approaches such as conjugation might be more accurate (Samperio et al., 2021).

Conjugation is a mechanism of horizontal gene transfer which occurs naturally and with high promiscuity. It allows the transfer of genetic material among all main bacterial types conferring high genomic plasticity to prokaryotes (De la Cruz and Davies, 2000). Recently, conjugation from *E. coli* to lactobacilli laboratory and wildtype strains using different conjugative systems has been described in our group. However, the efficiencies of conjugation obtained varied widely from the *L. casei* 393 laboratory strain which was around 10^{-3} or 10^{-4} transconjugants per donor, and the wildtype lactobacilli strains which were lower or not achievable (Samperio et al., 2021). An increase of the efficiencies could allow the detection of DNA mobilization in a broad range of bacteria. Considering the biomedical and biotechnological interest of this bacterial group, opening the way to introduce DNA in a wider number of species would be a significant achievement.

Our first goal was to increase the conjugation efficiency obtained from *E. coli* into lactobacilli, and for this, our approach was to modify the parameters from the protocol described to find the optimal ratio donor/recipient and the optimal donor and recipient growth phase. Conjugation was performed into *L. paracasei* BL23 using R388 and RP4 conjugative systems transferring the shuttle mobilizable plasmids pCOR48 and pCOR49 respectively. To test the influence of donor/recipient ratio on the conjugation efficiency, ratios ranging from 1000:1 to 1:100 donor/recipient (grown overnight) were used. No increase of the conjugation frequency was reported when variating the ratios, whereas a significant decrease was seen when there was 10 times more of either recipient or donor volume compared to the same amount (1:1). Then, with the optimal ratio 1:1 established, the role of the bacterial growth phase in conjugation was tested. Likewise, no significant optimization of the efficiency was reported when donors and recipients were on exponential growth phases compared to the stationary.

Since we were not able to optimize conjugation efficiency by modifying the protocol parameters, we conclude that conjugation frequency does not seem to increase when changing

donors or recipient's ratio, as well as performing the conjugation at bacterial stages different than stationary. However, it cannot be discarded that conjugation frequency would be optimized by a higher ratio of more than 10^3:1 or 1:10^2 (donors/recipients), although in the results obtained there is not a visible rise, but a general decrease. Studies examining the influence of donor/recipient ratio on conjugation were contradictory. In relation with our result, in (Lampkowska et al., 2008) the highest efficiency between lactobacilli conjugations was achieved when performing a 1:1 ratio, whereas in (Dominguez and O'Sullivan, 2013), ratios favoring *E. coli* were found to significantly increase conjugation to *Bifidobaterium* using RP4 system. Specifically, a ratio of 10^5:1 (donors/recipients) was reported to optimize transfer frequency per recipient. Also, ratios favoring the recipient bifidobacteria more than 1:10^2 didn't produce detectable transconjugants. In addition, the highest conjugation frequencies were reported with donors and recipients on the late-exponential growth phase, similarly to the result obtained in the present work. These differences between the results can be the determined by the donor, recipient and mobile elements used for the studies (Lampkowska et al., 2008).

Restriction-modification (RM) systems have been reported to impede foreign-DNA entry as a defense mechanism. This mechanism specifically detects the methylation pattern of incoming DNA, and restrict its entry if is recognized as non-self. Therefore, it has been described as partly responsible for the low transformation efficiencies obtained in wildtype strains. RM have been reported in some lactobacilli such as *L. plantarum*, for which successful transformation was archived introducing non-methylated DNA (Spath et al., 2012). Moreover, in previously untransformable *Staphylococcus* strains, non-cytosine methylated DNA entry was reported by a donor strain deficient in methylase (Monk et al., 2012). Then, we wondered if performing conjugation with this mutant strain as a donor would increase the conjugation efficiency.

In order to prove this, we introduced R388 and RP4 conjugative systems into the dcm-mutant strain with the corresponding shuttle mobilizable plasmids and performed conjugation into *L. paracasei* BL23. Conjugation frequency obtained was not significantly increased with R388 conjugative system. For RP4 conjugative system, we didn't observe any transconjugant colonies in any of the three replicas performed. Given this result, we conclude that conjugation frequency to *L. paracasei* BL23 is not increased when introducing non-methylated DNA. However, it is relevant to note that the use of the RP4 derivative pRL443 instead of the strain S17-1, which encodes the RP4 transfer system in its chromosome, led to a drastic reduction in transfer frequency also in the Dcm-proficient donor strain, which we can only explain by a defective pRL443. Experiments in the future using an efficient RP4 derivative for pCOR49 mobilization might render different results.

In addition, it should be pointed out that these restriction-modification systems and the successful transformation of lactobacilli with non-methylated DNA was described for wildtype

strains and the one we used, *L. paracasei* BL23, is a laboratory strain. For this reason, it could be interesting to perform the same conjugations but with a wildtype strain, such as *L. parabuchneri* as the recipient. Increasing optimization frequencies in wildtype lactobacilli strains could be a more interesting approach for the industry applications, as in some is not even feasible. This is the case for *L. parabuchneri,* an important bacterium affecting organoleptic properties of cheese, which has not been transformed yet, but can act as a conjugation recipient (Samperio *et al,* 2021).

After performing different approaches in order to optimize the conjugation efficiency and therefore, the introduction of DNA into lactobacilli, targeted genetic modification of this group was our main objective. For this, a homologous recombination cassette cloned in two different suicide or thermosensitive plasmids mobilizable into lactobacilli was designed to insert an erythromycin resistance gene in the place of the *lacG* gene in the chromosome. A simple approach that could be checked by plating transconjugants on selective media (MRS with Em) and on X-gal, so the colonies phenotype would be white as the *lacG* would not be functional. This design would allow us to obtain proof of concept for a novel useful tool for the genetic modification of wild type lactobacilli; although homologous recombination cassettes have been used in the past, the DNA has to be introduced by transformation, thus limiting the range of strains susceptible to genetic modification.

After several attempts of assembly of the different fragments, the desired construction was not obtained. Molecular analyses of the assembly reaction product revealed that not all fragments were joined, and specifically we could narrow the problem to the *em* fragment with one of the sequences of homology to *lacG* named *lacG$_2$* (assembly point 2). Then, sequencing analyses from these two fragments confirmed that the assembly was not happening as the joining point was incorrect. The reason is the existence of an unexpected homology region for one of the primers precisely close to the site where the complementary primer should bind, thus leading to a PCR product of the expected size but lacking the necessary tail on one side for assembly with the *lacG2* fragment.

Here, we conclude that even though the cassette was correctly designed, the assembly point 2 (erythromycin resistance gene with the second homology sequence for lactobacilli's genome) was not assembling as expected, thus precluding us from obtaining the desired products which would allow us to test the designed tool. Fortunately, we have been able to find out the problem. In order to successfully construct the complete plasmid, new primers should be designed for the amplification of the *em* fragment. This will allow to perform a new isothermal assembly and continue with the test for this potentially useful genetic modification tool for lactobacilli.

6. CONCLUSIONS

1. Conjugation frequency from *E. coli* to lactobacilli was not significantly increased when we varied either the donors or receptors ratio from $10^3:1$ to $1:10^2$.

2. No significant optimization of the conjugation efficiency was reported when donors and recipients were early or late exponential phases compared to stationary phase.

3. Conjugative transfer of methylation deficient DNA into *L. paracasei* BL23 did not significantly increase transfer frequency per donor.

4. We have designed an assay based on the use of bacterial conjugation from *E. coli* to *L. paracasei* to introduce a homologous recombination cassette for targeted mutagenesis.

5. The assembly of the complete homologous recombination cassette could be accomplished with the design of new primers for amplification of the correct Erythromycin fragment.

4. BIBLIOGRAPHY

Alvarez, M.A., Herrero, M., and Suárez, J.E. (1998). The Site-Specific Recombination System of theLactobacillusSpecies Bacteriophage A2 Integrates in Gram-Positive and Gram-Negative Bacteria. Virology *250*, 185-193.

Avila, P., and de la Cruz, F. (1988). Physical and genetic map of the IncW plasmid R388. Plasmid *20*, 155-157.

Aviv, G., Rahav, G., and Gal-Mor, O. (2016). Horizontal transfer of the Salmonella enterica serovar Infantis resistance and virulence plasmid pESI to the gut microbiota of warm-blooded hosts. MBio *7*, e01395-01316.

Ayivi, R.D., Gyawali, R., Krastanov, A., Aljaloud, S.O., Worku, M., Tahergorabi, R., da Silva, R.C., and Ibrahim, S.A. (2020). Lactic Acid Bacteria: Food Safety and Human Health Applications. Dairy *1*, 202-232.

Bernardeau, M., Vernoux, J.P., Henri-Dubernet, S., and Gueguen, M. (2008). Safety assessment of dairy microorganisms: the Lactobacillus genus. International journal of food microbiology *126*, 278-285.

Börner, R.A., Kandasamy, V., Axelsen, A.M., Nielsen, A.T., and Bosma, E.F. (2019). Genome editing of lactic acid bacteria: opportunities for food, feed, pharma and biotech. FEMS microbiology letters *366*, fny291.

Bosma, E.F., Forster, J., and Nielsen, A.T. (2017). Lactobacilli and pediococci as versatile cell factories–Evaluation of strain properties and genetic tools. Biotechnology advances *35*, 419-442.

Bradley, D.E. (1980). Morphological and serological relationships of conjugative pili. Plasmid *4*, 155-169.

Bron, P.A., Marcelli, B., Mulder, J., van der Els, S., Morawska, L.P., Kuipers, O.P., Kok, J., and Kleerebezem, M. (2019). Renaissance of traditional DNA transfer strategies for improvement of industrial lactic acid bacteria. Current opinion in biotechnology *56*, 61-68.

Buchanan-Wollaston, V., Passiatore, J.E., and Cannon, F. (1987). The mob and oriT mobilization functions of a bacterial plasmid promote its transfer to plants. Nature *328*, 172-175.

Cano-Garrido, O., Seras-Franzoso, J., and Garcia-Fruitós, E. (2015). Lactic acid bacteria: reviewing the potential of a promising delivery live vector for biomedical purposes. Microbial cell factories *14*, 1-12.

Costa, S.K., Donegan, N.P., Corvaglia, A.-R., François, P., and Cheung, A.L. (2017). Bypassing the restriction system to improve transformation of Staphylococcus epidermidis. Journal of bacteriology *199*.

Couturier, M., Bex, F., Bergquist, P.L., and Maas, W.K. (1988). Identification and classification of bacterial plasmids. Microbiological reviews *52*, 375.

Datta, N., and Hedges, R. (1972). Trimethoprim resistance conferred by W plasmids in Enterobacteriaceae. Microbiology *72*, 349-355.

De la Cruz, F., and Davies, J. (2000). Horizontal gene transfer and the origin of species: lessons from bacteria. Trends in microbiology *8*, 128-133.

de Vos, W.M. (2011). Systems solutions by lactic acid bacteria: from paradigms to practice. Microbial Cell Factories *10*, 1-13.

Demarre, G., Guérout, A.-M., Matsumoto-Mashimo, C., Rowe-Magnus, D.A., Marlière, P., and Mazel, D. (2005). A new family of mobilizable suicide plasmids based on broad host range R388 plasmid (IncW) and RP4 plasmid (IncPα) conjugative machineries and their cognate Escherichia coli host strains. Research in microbiology *156*, 245-255.

Derkx, P.M., Janzen, T., Sørensen, K.I., Christensen, J.E., Stuer-Lauridsen, B., and Johansen, E. (2014). The art of strain improvement of industrial lactic acid bacteria without the use of recombinant DNA technology. Microbial cell factories *13*, 1-13.

Dominguez, W., and O'Sullivan, D.J. (2013). Developing an efficient and reproducible conjugation-based gene transfer system for bifidobacteria. Microbiology *159*, 328-338.

Elhai, J., Vepritskiy, A., Muro-Pastor, A.M., Flores, E., and Wolk, C.P. (1997). Reduction of conjugal transfer efficiency by three restriction activities of Anabaena sp. strain PCC 7120. Journal of bacteriology *179*, 1998-2005.

Fernández-López, R., Garcillán-Barcia, M.P., Revilla, C., Lázaro, M., Vielva, L., and De La Cruz, F. (2006). Dynamics of the IncW genetic backbone imply general trends in conjugative plasmid evolution. FEMS microbiology reviews *30*, 942-966.

Francia, M.V., Varsaki, A., Garcillán-Barcia, M.P., Latorre, A., Drainas, C., and de la Cruz, F. (2004). A classification scheme for mobilization regions of bacterial plasmids. FEMS microbiology reviews *28*, 79-100.

Getino, M., and de la Cruz, F. (2019). Natural and artificial strategies to control the conjugative transmission of plasmids. Microbial Transmission, 33-64.

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C.A., and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nature methods *6*, 343-345.

Grohmann, E., Muth, G., and Espinosa, M. (2003). Conjugative plasmid transfer in gram-positive bacteria. Microbiology and molecular biology reviews *67*, 277.

Haase, J., Lurz, R., Grahn, A.M., Bamford, D.H., and Lanka, E. (1995). Bacterial conjugation mediated by plasmid RP4: RSF1010 mobilization, donor-specific phage propagation, and pilus production require the same Tra2 core components of a proposed DNA transport complex. Journal of bacteriology *177*, 4779.

Heinemann, J.A., and Sprague, G.F. (1989). Bacterial conjugative plasmids mobilize DNA transfer between bacteria and yeast. Nature *340*, 205-209.

Horvath, P., and Barrangou, R. (2010). CRISPR/Cas, the immune system of bacteria and archaea. Science *327*, 167-170.

Isolauri, E., Salminen, S., and Ouwehand, A.C. (2004). Probiotics. Best practice & research Clinical gastroenterology *18*, 299-313.

Killmann, H., Benz, R., and Braun, V. (1996). Properties of the FhuA channel in the Escherichia coli outer membrane after deletion of FhuA portions within and outside the predicted gating loop. Journal of Bacteriology *178*, 6913-6920.

Klaenhammer, T.R., Barrangou, R., Buck, B.L., Azcarate-Peril, M.A., and Altermann, E. (2005). Genomic features of lactic acid bacteria effecting bioprocessing and health. FEMS Microbiology Reviews *29*, 393-409.

Lampkowska, J., Feld, L., Monaghan, A., Toomey, N., Schjørring, S., Jacobsen, B., van der Voet, H., Andersen, S.R., Bolton, D., and Aarts, H. (2008). A standardized conjugation protocol to asses antibiotic resistance transfer between lactococcal species. International journal of food microbiology *127*, 172-175.

Llosa, M., and de la Cruz, F. (2005). Bacterial conjugation: a potential tool for genomic engineering. Research in microbiology *156*, 1-6.

Makarova, K.S., Wolf, Y.I., Iranzo, J., Shmakov, S.A., Alkhnbashi, O.S., Brouns, S.J., Charpentier, E., Cheng, D., Haft, D.H., and Horvath, P. (2020). Evolutionary classification of CRISPR–Cas systems: a burst of class 2 and derived variants. Nature Reviews Microbiology *18*, 67-83.

Martín, M.C., Alonso, J.C., Suárez, J.E., and Alvarez, M.A. (2000). Generation of food-grade recombinant lactic acid bacterium strains by site-specific recombination. Applied and Environmental Microbiology *66*, 2599.

Martín, M.C., Fernández, M., Martín-Alonso, J.M., Parra, F., Boga, J.A., and Alvarez, M.A. (2004). Nisin-controlled expression of Norwalk virus VP60 protein in Lactobacillus casei. FEMS microbiology letters *237*, 385-391.

Martinez, E., and de la Cruz, F. (1988). Transposon Tn 21 encodes a RecA-independent site-specific integration system. Molecular and General Genetics MGG *211*, 320-325.

Mays, Z.J., and Nair, N.U. (2018). Synthetic biology in probiotic lactic acid bacteria: At the frontier of living therapeutics. Current opinion in biotechnology *53*, 224-231.

Mazé, A., Boël, G., Zúñiga, M., Bourand, A., Loux, V., Yebra, M.J., Monedero, V., Correia, K., Jacques, N., and Beaufils, S. (2010). Complete genome sequence of the probiotic Lactobacillus casei strain BL23. Journal of bacteriology *192*, 2647-2648.

Monk, I.R., and Foster, T.J. (2012). Genetic manipulation of Staphylococci–Breaking through the barrier. Frontiers in cellular and infection microbiology *2*, 49.

Monk, I.R., Shah, I.M., Xu, M., Tan, M.-W., and Foster, T.J. (2012). Transforming the untransformable: application of direct transformation to manipulate genetically Staphylococcus aureus and Staphylococcus epidermidis. MBio *3*.

Ouwehand, A.C., Salminen, S., and Isolauri, E. (2002). Probiotics: an overview of beneficial effects. Lactic acid bacteria: genetics, metabolism and applications, 279-289.

Plavec, T.V., and Berlec, A. (2020). Safety aspects of genetically modified lactic acid bacteria. Microorganisms *8*, 297.

Sadler, J.R., Tecklenburg, M., and Betz, J.L. (1980). Plasmids containing many tandem copies of a synthetic lactose operator. Gene *8*, 279-300.

Samperio, S., Guzmán-Herrador, D.L., May-Cuz, R., Martín, M.C., Álvarez, M.A., and Llosa, M. (2021). Conjugative DNA transfer from E. coli to transformation-resistant lactobacilli. Frontiers in Microbiology *12*.

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. Bio/technology *1*, 784-791.

Song, L., Cui, H., Tang, L., Qiao, X., Liu, M., Jiang, Y., Cui, W., and Li, Y. (2014). Construction of upp deletion mutant strains of Lactobacillus casei and Lactococcus lactis based on counterselective system using temperature-sensitive plasmid. Journal of microbiological methods *102*, 37-44.

Song, X., Zhang, X.-y., Xiong, Z.-q., Liu, X.-x., Xia, Y.-j., Wang, S.-j., and Ai, L.-z. (2020). CRISPR–Cas-mediated gene editing in lactic acid bacteria. Molecular Biology Reports, 1-12.

Spath, K., Heinl, S., and Grabherr, R. (2012). Direct cloning in Lactobacillus plantarum: electroporation with non-methylated plasmid DNA enhances transformation efficiency and makes shuttle vectors obsolete. Microbial cell factories *11*, 1-8.

Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N., and Court, D.L. (2014). Recombineering: genetic engineering in bacteria using homologous recombination. Current protocols in molecular biology *106*, 1.16. 11-11.16. 39.

Trieu-Cuot, P., Carlier, C., and Courvalin, P. (1988). Conjugative plasmid transfer from Enterococcus faecalis to Escherichia coli. Journal of Bacteriology *170*, 4388-4391.

Vasu, K., and Nagaraja, V. (2013). Diverse functions of restriction-modification systems in addition to cellular defense. Microbiology and molecular biology reviews *77*, 53-72.

Walker, D., and Klaenhammer, T. (1994). Isolation of a novel IS3 group insertion element and construction of an integration vector for Lactobacillus spp. Journal of Bacteriology *176*, 5330.

Wang, C., Cui, Y., and Qu, X. (2020). Optimization of electrotransformation (ETF) conditions in lactic acid bacteria (LAB). Journal of microbiological methods, 105944.

Waters, V.L. (2001). Conjugation between bacterial and mammalian cells. Nature genetics *29*, 375-376.

Yasui, K., Kano, Y., Tanaka, K., Watanabe, K., Shimizu-Kadota, M., Yoshikawa, H., and Suzuki, T. (2009). Improvement of bacterial transformation efficiency using plasmid artificial modification. Nucleic acids research *37*, e3-e3.

Zechner, E., De La Cruz, F., Eisenbrandt, R., Grahn, A., Koraimann, G., Lanka, E., Muth, G., Pansegrau, W., Thomas, C., and Wilkins, B. (2000). Conjugative-DNA transfer processes. The horizontal gene pool: bacterial plasmids and gene spread *23*, 419.

Zheng, J., Ruan, L., Sun, M., and Gänzle, M. (2015). A genomic view of lactobacilli and pediococci demonstrates that phylogeny matches ecology and physiology. Applied and environmental microbiology *81*, 7233-7243.

Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M., Harris, H.M., Mattarelli, P., O'Toole, P.W., Pot, B., Vandamme, P., and Walter, J. (2020). A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. International journal of systematic and evolutionary microbiology *70*, 2782-2858.

Zupan, J., Ward, D., and Zambryski, P. (2002). Inter-kingdom DNA transfer decoded. Nature biotechnology *20*, 129-131.