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Identifying Genes that Affect Lactococcus lactis Intron Mobility

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"Identifying Genes that Affect *Lactococcus lactis* Intron Mobility"

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Abstract

A gene consists of coding segments, exons, which are maintained and contribute to the final mRNA product, while non-coding segments, introns, are spliced out through RNA splicing mechanisms to code a functional gene. The mobility of the spliced introns and factors that affect mobility are an important field of study evolutionarily whereas splicing defects have medical implications. For this study, the genome of the *Lactococcus lactis* bacterial strain, IL1403 and the group II intron of interest, L1.LtrB were utilized. This intron moves into new sites on DNA by a process termed retrotransposition (RTP). Our goal is to identify genes that regulate retrotransposition of L1.LtrB in *L. lactis*. To do this, we are making a transposon mutant library in *L. lactis* strain IL1403. The pG+host::IS*S1* plasmid was used to introduce the IS*S1* transposon into the genome of IL1403 at random positions, creating a library of mutants. The intron donor plasmid, containing the Ll.LtrB intron was also introduced into IL1403 and later used for retrotransposition assays that will determine the degree by which certain genes affect Ll. LtrB intron mobility. We have also developed a method to screen mutants for RTP levels via hybridization and selective media. So far we have generated over 1000 mutants with several showing stimulatory or inhibitory effects on retrotransposition. Genetic recombineering will follow allowing recreation of mutants for further analysis. This will occur after high-throughput sequencing which will localize specific genes that will give clues as to which host facts increase or decrease retrotransposition.

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Introduction

A bacterial genome consists of a single circular chromosome that contains all the bacteria's genetic information. The genes within a genome determine how bacteria function in response to internal and external environmental cues. A few genes are interrupted and contain coding segments, exons, and non-coding segments, introns. These introns go through a process called RNA splicing in which they are spliced out of the gene, leaving behind a continuous functional gene.

The intron I am studying is also a retrotransposon that codes for reverse transcriptase (RT). RT is also coded for by retroviruses like HIV and telomerases at the ends of chromosomes. Therefore, what I learn from these bacterial introns and their relationship to the host will inform studies on these important eukaryotic retroelements that help shape genomes. Our bacterial genome of interest is *Lactococcus lactis*. *L. lactis* is a gram-positive bacterium used in the production of dairy products. The specific strain of *L. lactis* used in this experiment is IL1403. Our intron of interest is Ll. LtrB, a 2.5 kb group II intron carried on the conjugative element pRS01. Group II introns are mobile retro-elements that invade their associated intron-minus gene by retrohoming (Figure 1A) (Coros *et. al*., 2005). Group II introns are also identified as retrotransposons as they can move to ectopic sites by retrotransposition (Figure 1B) (Beauregard *et al.,* 2008). Once an intron is exised, it forms an intron lariat which retrotransposes into ectopic sites. Retrotransposition is the movement of genetic elements to ectopic sites in the genome via an RNA intermediate. Mobility of an intron can be dependent on host factors as well as environmental factors (Coros *et al.*, 2008, Beauregard *et al*., 2008).

Ll. LtrB, our intron of interest, will be introduced to the cell on an intron donor plasmid pLNRK-RIG. The plasmid pLNRK-RIG (*r*etrotransposition *i*ndicator *g*ene*)*, will be our model for detecting retrotransposition since it indicates retrotransposition events directly (Figure 2A) (Ichiyanagi *et al.*, 2002). Within the donor plasmid, the intron Ll. LtrB carries a gene that encodes for kanamycin antibiotic resistance (Kan^R), interrupted by a small group I intron. The lab has developed a retrotranspositon assay where the Kan gene is engineered into the intron. Only upon splicing of this group I intron while going through an RNA intermediate will we get Kanamycin resistance (Figure 2B). The Kan^R marker is used to indicate intron mobility events (Cousineau *et al*., 2000). In addition, plasmid pLNRK-RIG backbone has chloramphenicol resistance (Cam^R). The intron donor plasmid pLNRK-RIG will remain as a circular plasmid throughout the IS*S1* mutagenesis protocol so the use of Cam media will select for maintenance of the RIG plasmid.

The goal of our experiment is to identify host genes which affect retrotransposition by looking at host mutants of the IL1403/pLNRK-RIG strain. A similar mutant screen was done in a previous study with *Escherichia coli* (Coros *et al.* 2008). We aim to create a mutant library to be able to select mutants that affect retrotransposition in *L.lactis*, the natural host of the Ll. LtrB intron. There are several methods of creating a mutant library such as using the Mu phage transposon or the Tn5 transposon. However we used the temperature-sensitive plasmid pG+host::IS*S1* (Figure 3) (Maguin *et al.* 1996). The pG+host plasmid, a circular plasmid, was transformed into the IL1403/pLNRK-RIG cell. Plasmid pG+host::IS*S1* contains a temperature sensitive (ts) element that allows pG+host to be "cured" because the plasmid is able to replicate at 28ºC and fails to replicate at 37.5ºC. The plasmid also contains a gene that encodes for erythromycin resistance (Erm^R), which allows curing to be evaluated after temperature upshift.

The use of pG+host was important in this experiment since it was the delivery vector of the gene *ISS1* (Maguin *et al.* 1996). *ISS1* is used to perform mutagenesis as it will randomly insert itself along with the plasmid backbone in a way that it is duplicated on either side of the pG+host sequence in the genome of IL1403/pLNRK-RIG allowing us to select for transposition of *ISS1*. Then through temperature shifts, we can stimulate the plasmid backbone to excise from the genome, leaving only one copy of IS*S1* in the genome. Finally, we can then shift temperature to remove reistant pG+host plasmid from the cell. These mutants can then be further verified and used in screens for RTP effects.

Overall this experiment established a method of screening for genes that affect retrotransposition using the transposon IS*S1* for mutant library preparation. These experiments will be important for determining retroelement-host relationships and inform ongoing work with retroelements in eukaryotes. Since group II introns are thought to be the progenitors of spliceosomal introns, studying their behaviors can unfold valuable information on the evolution of eukaryotes. The research on understudied organisms such as *Lactococcus lactis* contributes to the understanding of bacteria as a whole, while defining the functioning of an organism that is of great intesert to the dairy industry.

Materials and Methods

Bacterial strains. *Lactococcus lactis* strain IL1403. pLNRK-RIG plasmid and pG+host plasmid, containing the IS*S1* transposon were used in this study. *L. lactis* strains were grown in M17 media (BD 218561) supplemented with 0.5% glucose containing appropriate antibiotics. The plasmid pJP005 containing recT was used for recombineering experiments. NZ9000 was used for recombineering and contains *nisR nisK* genes needed for expression in pJP005. E.coli culture DH5 was grown in LB media (BD 244620). pGEM plasmid was used for subcloning the *nisRK* fragment. Oligos used in this study are shown in Table I.

Plasmid preparations. To prepare our strain for mutagenesis, *L.lactis* was transformed with plasmid pLNRK-RIG. For plasmid extraction, a Qiagen Miniprep Kit (27106) was used. An overnight culture was pelleted and resuspended in 250 µl of buffer P1 (50mM Tris-HCl, 10mM EDTA, 100µg/ml RNaseA) then transferred to microcentrifuge tube. Then 250 µl of buffer P2 (200 mM NaOH, 1% SDS) was mixed by inverting until solution was clear. Next, 350 µl of buffer N3 (4.2 M Gu-HCl and 0.9 M potassium acetate) was added and the solution was mixed again by inverting. The solution was centrifuged for 10 minutes at 13,000 rpm. The supernatant was poured onto Qiagen columns with collection tubes and centrifuged for 60 seconds. Then 500 µl of buffer PB (5M Gu-HCL,30% isoproponal) was added and centrifuged for 30 to 60 seconds; the flow-through was discarded. Next, 750 µl of buffer PE (10mM Tris-HCl, 80% ethanol) was added, and the flow- through was discarded. The column was put back in the collection tube and spun to remove residual EtOH. Then 50 µl of water was added to the column and centrifuged to elute the DNA. We then ran an agarose gel to verify the presence of the plasmid.

Electro-competent cells. Competent cells of IL1403 were made so that we can electroporate or transform the PLNRK-RIG and pG+Host::IS*S1* plasmids into the IL1403 cell. Electrocompotent cells were made by growing an overnight cultures of IL1403 in GM17 media, diluting 1:100, spinning the cells down (discarding supernatant) and finally doing three washes with EP buffer (0.5 M sucrose, 10% glycerol). The final pellet was resuspended in 200- 300 µl then 40 µl aliquots were stored at -70ºC.

Electrotransformation of plasmids into IL1403. The pLNRK-RIG plasmid was transformed into IL1403 competent cells. One microliter of plasmid preparation placed in chilled 2mm cuvettes with 40 µl competent cells. The cells were given a pulse of 2000 V (200 Ω, 2 µF) using a BioRad electroporator. The cells were immediately transferred to rich GM17 media with 0.5% glucose and 0.5 M sucrose in a 1.5 ml Eppendorf tube. The cell suspension was allowed to recover for 3 hours at 30ºC. The cultures were spun down and most of the supernatant was discarded. The pellet was resuspended in residual media and spread onto an agar plate containing Cam_{10} (chloramphenicol antibiotic 10µg/ml) and incubated overnight at 30ºC.

The next step was to transform pG+host::IS*S1* into IL1403/pLNRK-RIG strain in a similar method as above, but the transformation was plated on Erm_5 plates. Any resulting transformants were confirmed to also be resistant to Cam_{10} (maintained pLNRK-RIG).

ISS1 transposition to generate mutants. An overnight culture of IL1403/pLNRK-RIG/ pG+host::IS*S1* was diluted 1:100 in Cam_{10} media and incubated at 28ºC for 150 minutes. Then the culture was shifted to 37.5ºC for 150 minutes. Finally 100 µl of 10^{-1}, 10^{-2} and 10^{-3} dilutions of the culture were plated onto Cam_5Erm_2 (erythromycin antibiotic 2µg/ml) plates and incubated at 37º C.

The colonies that grew on the Cam_5Erm_2 plate were used to inoculate a 96-well plate of cultures. The 96-well plate was incubated at 37ºC overnight. A second 96-well plate was generated the following day or until cells were grown to saturation, by diluting each well $^1/_{1000}$ into fresh media. This plate was incubated at 28ºC for 18 hours. Next, to isolate single colonies 6-well plates were generated with each respective culture from the 96-well plate. One microliter from each well was pipetted onto a 6 well plate to streak for single colonies. The 6-well plates were incubated at 37ºC.

The single colonies grown on the 6-well plate were then patched on a Cam_5 agar plate. These plates were designed in a "wagon wheel"; the plate was divided into 12 sectors, and each of the 6 wells were patched 3 times in their sector of the plate (Figure 3). This "wagon wheel" plate was incubated at 37ºC overnight, and then replica plated onto Erm_2, then Cam_5 plates.

After the overnight incubation of the "wagon wheel" plate, replicas of this plate were made. The original "wagon wheel" plate was set on a velvet cloth then its imprints were transferred to an Erm_2 plate then a Cam_5 plate. The replica plates were incubated at 37ºC overnight. After the incubation, potential cured mutants that did not grow on the Erm_2 plates (pG+host negative) but did grow on the Cam_5 plates were selected (pLNRK-RIG positive) were selected. We then used one of two methods to verify the mutants. The two methods were PCR of genomic DNA and radioactive probing to verify transposon (IS*S1)* and pLNRK-RIG plasmid presence.

***Genomic DNA preparation*.** Genomic DNA was prepared from potential mutants using a DNeasy Blood and Tissue Kit from Qiagen (69504). First cells were harvested in a microcentrifuge tube by centrifuging for 10 minutes at 7500 rpm, the supernatant was discarded.

Then the bacterial pellet was resuspended in 180 µl of enzymatic lysis buffer consisting of 20 mM Tris-Cl (pH 8), 2 mM sodium EDTA, 1.2% Triton X-100, 20 mg/ml of lysozyme.

The cleared lysate was then incubated for 30 minutes at 37ºC. Next 25 µl of proteinase K and 200 µl of Buffer AL (without ethanol) were added, and mixed by vortexing. A second incubation at 56ºC was carried out for 30 min. Then 200 µl of ethanol was added to the sample and mixed thoroughly by vortexing. The mixture was then pipetted in a DNeasy Mini spin column and placed in a 2 ml collection tube, followed by centrifugation at 8000 rpm for 1 minute. All flow-through along with the collection tube was discarded.

The DNeasy Mini spin column was placed in a new 2ml collection tube along with 500 µl of Buffer AW1 and centrifuged for 1 minute then Buffer AW2, and centrifuged for 3 minutes at 14,000 rpm. Once again the flow-through and the collection tube were discarded. Next the DNeasy mini spin column was placed in a clean 2 ml microcentrifuge tube and 200 µl of DI water was pipetted directly onto the DNeasy membrane; after incubation at room temperature for 1 minute the mixture was centrifuged for one minute. The purified DNA was then used for PCR.

PCR reactions. PCR reactions were done to verify IS*S1* transposon and RIG plasmid presence. In the initial step of PCR the target DNA is denatured by heating to 95ºC for 3 minutes. In the next step, the temperature was reduced to 55ºC for primer annealing. In the last step, the temperature was raised to 72ºC to begin DNA synthesis. Thirty cycles of PCR were done with a final extension time of 10 minutes. The PCR products then underwent agarose gel electrophoresis for size separation of the products. The sizes of the products were compared to a 1kb ladder.

The primers to amplify IS*S1* were IDT 3705 and IDT 3706. In the tube 2 µl of genomic DNA, 5µl of IDT 3705, 5 µl of IDT 3706, 25 µl of 2x PCR mix and 13 µl of H_2O was added.

Primers to amplify RIG were IDT 374 and IDT 375. In the tube 2µl of genomic DNA, 5µl of IDT 374, 5 µl of IDT 375, 25 µl of 2x PCR mix and 13 µl of H_2O was added. Both the IS*S1* and RIG PCR mixes underwent PCR, testing for the presence of IS*S1*and RIG in the mutants. Each mutant underwent both PCR's.

Radioactive probing. Mutants that were not put through PCR underwent radioactive probing. First, we did colony lifts for all the mutants. A marked membrane was placed on each plate with patches of potential mutants, and left on for 5 minutes. The membrane was placed colony side up on filter paper. Puddles of 750 µl of 30 mg/ml lysozyme were placed on a sequencing plate. The membranes were placed colony side up on the puddle and incubated twice for 15 minutes each at 37ºC. The membranes were then placed colony side up on filter paper to briefly dry. Next the sequencing plate was wiped down by spraying it with water and ethanol. Then the colonies were bathed in 750 µl puddles of three solutions. The membranes were placed in a puddle of solution 1, 0.5M NaOH, for two 3 minute increments. The membranes were placed in a puddle of solution 2, 1 M TrisCl, for two 5 minute increments. Lastly, the membranes were placed in a puddle of solution 3, 1.5 M NaCl and 0.5 M TrisCl, for one 5 minute increment. The membranes were then allowed to dry for 30 to 60 minutes. With the colony side up the membranes were placed in the UV crosslinker to link DNA to filters. The membranes were packed in saran wrap, and stored at 4ºC. Next, the membranes were hybridized with radiolabeled IS*S1* probe and a RIG probe. Only mutants with a positive signal for both IS*S1* and RIG were verified as part of our mutant library.

Sequencing mutants using inverse PCR. Some mutants that were IS*S1* positive and RIG positive were identified by sequencing. Our sequencing strategy was with the use of inverse PCR and the restriction enzyme, Sau3A1 (Figure 4).

We used inverse PCR to identify the gene that was interrupted by the IS*S1* transposon (Ochman *et al.,* 1988). This was done after digestion of our genomic DNA utilizing the Sau3A1 restriction enzyme and subsequent recircularization by DNA ligase. The resulting 5' and 3' circles acted as a substrate for amplification by PCR. Unlike conventional PCR, Inverse PCR goes out around the ligated circles. By selecting Sau3A1 to cut within IS*S1* and the IL1403 genome and using inverse PCR the upstream and downstream flanking regions will be produced.

First genomic DNA of mutant was digested with Sau3A1. A mix of 10µl of genomic DNA, 2 µl of NEB1 buffer, BSA buffer, 4.5 µl of H_2O and 1.5µl of Sau3A1 was prepared in a 1.5 ml tube. This mixture was incubated at 37º C overnight. To heat inactivate the Sau3A1 enzyme, to prevent further cutting of the genome, the digest was incubated at 65º C for 20 minutes. At this point the genomic DNA with the IS*S1* transposon was cut into fragments and ligation was carried out next. A mix of 3µl of digested genomic DNA, 1 µl of 10x T4 DNA ligase buffer, 1 µl of T4 DNA ligase and 5 µl of H_20 was added into a new 1ml tube. Ligase was added to re-circularize the previously digested DNA. This sample was incubated at 16º C overnight. Then incubated at 65º C for 20 minutes to heat inactivate ligase.

PCR was then used to amplify flanks of interest from circularized DNA. For the 5' flank, primer IDT 3858 and primer IDT 3859 were used. For the 3' flank IDT 3860 and primer IDT 3861 were used. After the samples underwent PCR, they were loaded onto a 1% agarose gel and observed.

PCR Purification. Those mutants that underwent PCR were put through PCR purification to remove impurities from the samples. A QIAquick PCR Purification Kit was utilized. First 200 µl of Buffer PB was added to 40µl of the PCR sample. The sample and Buffer PB was added to a QIAquick column in a 2 ml collection tube. In order to bind the DNA the

sample and Buffer PB were centrifuged for 60 seconds at 13000 rpm; the flow through was discarded and the QIAquick column was placed back in the same tube. Then to wash to DNA, 750 µl of Buffer PE was added to the QIAquick column and was centrifuged for 60 seconds at 13000 rpm. Once again the flow through was discarded and the QIAquick column was placed back into the same tube. The QIAquick column was centrifuged once again in the same 2 ml collection tube for 1 minute at 13000 rpm to rid the column of remaining wash buffer. The QIAquick column was then placed in a clean 2 ml collection tube. To then elute the DNA, 50 µl of water was added to the QIAquick column and centrifuged for 1 minute at 13000 rpm. Samples were left to stand in the column for 1 minute.

After purification, the mutants were sent to Eton Biosciences for sequencing. The 5' samples and the 3' samples were kept separate to sequence from both ends. Primer 1, IDT 3858, was used to identify sequences on the 5' end and primer 4, IDT 3861, for sequences on the 3' end (Figure 4C).

Retrotransposition (RTP) assay (pooling of mutants and high throughput sequencing). Overnight cultures of IL1403:IS*S1*/RIG mutants were set up in a 96 deep well plate. Each well contained 1 ml of GM17 Cam_{10} media and was inoculated with a small amount of glycerol stock. Additionally overnights of controls, IL1403/pLNRK-RIG (Cam_{10}) and IL1403/pLNRK-RIG/pRS01 (Cam_{10} + Erm_{10}), were set up. All cultures were incubated at 30ºC overnight. The following morning using a multichannel pipetman, 100 µl of mutants from each well were combined in a reservoir and then transferred into a Falcon tube. Then 300µl overnight culture mix was added to 10ml fresh GM17 Cam_{10} media, making a 1/30 dilution. The mutant and control cultures were grown until OD_{600} reached around 0.2. The cultures were then induced for three hours using 10 ng/ml concentration of nisin. Dilutions of IL1403:IS*S1*/RIG cultures were

plated on GM17 plates containing different Kan antibiotic concentrations (0, 40, 80 and 160 μg/ml). Different Kan concentrations were used to select for RTP events, different resistance phenotypes. GM17 media was used for growth while kanamycin media was used to detect if a retrotransposition event occurred. First the mutants were plated on GM17 media, with the mutants diluted to the 10^{-4}, 10^{-5}, and 10^{-6} [here forth referred to as -4,-5 and -6]. Next, they were plated on GM17 Kan_{40} media with dilutions to the -2, -3, and -4. Then mutants were plated on GM17 Kan_{80} media with dilutions to the -1, -2 and -3. Lastly the mutants were plated on GM17 Kan_{160} with dilutions to the -1 , -2, and one undiluted batch of mutants. The control was plated on GM17 media (-4,-5 and -6) and Kan_{160} (UD, -1 and -2). Each dilution was plated on three plates of the same media. This was done so that an average of the number of colonies could be taken. All of the plates for both the IL1403:IS*S1*/RIG mutants and IL1403/RIG control were grown at 30ºC over two nights. Then the colonies were counted to determine the RTP frequencies for the different Kan concentrations relative to that without antibiotic. Colonies were scooped from plates that had about 100-500 colonies and not taken from those that grew into lawns. Each type of media was scooped separately. To scoop the colonies 2-3ml of GM17 was first pipetted onto the plate then the colonies were mixed in the media and transferred to the next plate, until all colonies were scooped from the set of 3 plates. All scooped colonies were transferred into a 1.5ml eppendorf tube, spun down, the supernatant was removed and then the pellet was frozen at -80ºC.Genomic DNA was prepared and DNA was sheared and further processed for high throughput sequencing.

RTP Assay (screening). Mutants were assayed individually (as above) in 96-well plates. After induction cultures were spotted onto filters for subsequent hybridization with RIG splice junction probe (the complementary sequence occurs only in successful RTP events) to measure

the level of RTP and *L. lactis* probes to ensure that the strains are growing at similar rates so that RTP levels can be accurately compared. They were also spotted on selective Kan_{160} plates to select for RTP. The hybridization was more useful in identifying inhibitory mutants while the selective media was more useful in identifying stimulatory mutants. The mutants of interest will eventually all be sequenced to determine which genes affect retrotransposition.

Recombineering. In work by van Pijkeren and Britton recombineering was successful in NZ9000 *L.lactis* strain so we hope to develop a similar system in IL1403 which lacks the nisR nisK genes to drive expression of the *recT* gene necessary for single strand recombineering (van Pijkeren and Britton 2012). The IL1403/pJPOO5 (recT) +nisRnisK system was constructed by first amplifying the nisR nisK insert from the pLNRK plasmid using primers IDT 4320 and IDT 4321 and Accuprime pfx polymerase (Invitrogen). The pLNRK plasmid underwent PCR for the nisR nisK insert to be amplified. The PCR products were run out on an agarose gel and purified using a gel extraction kit and a PCR purification kit. The pJP005 vector was purified and linearized so it could be used for our cloning mechanism. After purification the insert was subcloned into pGEM vector. pGEM cloning required the addition of "A" overhangs to the PCR of the insert. A master PCR mix containing T_{aq} polymerase and water was added. This mix underwent PCR then was purified using a Qiagen PCR purification kit (28704). The pGEM vector along with the insert were ligated by making a mix with ligation buffer, T4 DNA ligase and water incubated at room temperature for an hour. Then 2 µl of the ligation was transformed into electrocompetent DH5α cells and recovered in SOC media and incubated for 37°C for 1 hour. The transformation (100 µl) was plated on LB+Amp plates incubated overnight at 37°C. The following day, a Qiagen Miniprep Kit was used to miniprep several single colonies from the pGEM+ nisRnisK PCR cloning. The *nisR nisK* insert contains BglII sites on both sides which

can be used as a tool to cut nisR nisK out of vectors. A Bgl II digestion was performed using BglII enzyme mixed with the pGEM plasmid DNA, NEBuffer and water. After the digest with the BglII restriction enzyme a gel was run, and favorable candidates were inoculated into LB media overnight. Then an agarose gel, followed by gel purification, was run to separate the *nisR nisK* insert from the pGEM vector. To get our insert into the pJP005 vector a ligation was done followed by a transformation of the ligation into *L. lactis* MG1363 Transformants were then plated and incubated at 30°C overnight.

Results

pG+host::ISS1 mutagenesis: Our goal was to create a mutant library in IL1403 *L. Lactis* that we can use to screen for host factors that stimulate or inhibit RTP. ILl403 cells containing pG+host::IS*S1* and pLNRK-RIG were grown, undergoing temperature shifts to insert *ISS1* in the genome. IS*S1* mutants were selected on plates containing GM17 media with Cam_5Erm_2 (chloramphenicol antibiotic 5µg/ml and erythromycin antibiotic 2µg/ml) then each mutant was kept separate in a deep well plate. After several temperature shifts to excise pG+host plasmid from the genome and remove circular plasmid from the cell, mutants were selected and verified. We have created over 1000 mutants to date.

Plasmid pG+host::*ISS1* delivers *ISS1* to the genome of IL1403/pLNRK-RIG allowing us to select for transposition of *ISS1* at the non-permissive temperature (Maguin *et al.* 1996). At 37.5ºC, the circular form of pG+host plasmid is lost due to the temperature sensitive replicon and the *L.lactis* strain will now only have pG+host sequence in the genome. When the mutants were grown on Cam_5 "wagon wheel" plates then patched to Erm_2 and Cam_5 replica plates our potential mutants came out to be Erm sensitive. This means that pG+host plasmid was lost and Cam resistant meaning pLNRK-RIG plasmid was maintained (Figure 3). The two methods for the verification of mutants were, polymerase chain reaction (PCR) on genomic DNA and radioactive probing for IS*S1* and RIG.

PCRs to confirm ISS1 transposon and pLNRK-RIG plasmid*:* Genomic DNA was prepared for PCR to determine if our mutants contained both IS*S1* and RIG in their DNA with the primers we designed. We selected mutants that gave bands on the gel for both the IS*S1* PCR and the RIG PCR.

Figure 4A (left) shows the results of the IS*S1* PCRs. The first lane is a 1kb plus ladder, the second was the negative control with no template DNA, the third was the negative control of IL403 genomic DNA, the fourth was a positive control of pGhost4::IS*S1* and in the fifth row a negative control of pLNRK-RIG was used. The last five rows are our potential mutants (lanes 1-5). Our controls worked, the second lane with no template DNA came out negative showing no band, the third lane with IL1403 genomic DNA, which was a negative control, showed no band, which was because IS*S1* had not been inserted prior. The positive control, pG+host4::IS*S1*, showed a band since this control contained IS*S1*. Overall the majority of our sample mutants came out to be IS*S1* positive.

Figure 4A (right) also shows results for the RIG PCR to verify the presence of the pLNRK-RIG plasmid. A 1 kb ladder was used as a size marker. The lane with no template DNA showed no band,which was expected. The positive control pLNRK-RIG had a band, meaning it was RIG positive. The negative control, pG+Host4::IS*S1*, did not show a band so it was RIG negative. It was evident that all of the test samples showed RIG positivity (lanes 1-5).

Colony hybridization: Radiolabeling is also a way to verify the mutants; it is also a method that allows a multitude of mutants to be analyzed simultaneously. First, colony lifts were done to bind mutant patches to a membrane, which is later used for hybridization. The membranes were probed with an IS*S1* probe to see if IS*S1* is present and the mutant is IS*S1* positive, and a probe for RIG was used to determine if pLNRK-RIG is present. Colony hybridization was useful analyzing a larger number of mutants. Mutants that were CamR and ErmS were taken from a Cam master plate.

In Figure 4B darker patches show positivity for either IS*S1* or RIG. The + denotes the positive control, and – denotes the negative control. Those patches that have a lighter patch were

negative for IS*S1* and RIG. About 50% of the patches showed IS*S1* positivity and RIG (Kan) positivity.

DNA Sequencing: When the correct mutants are verified, sequencing of selected mutants was done. The purpose of sequencing is to determine where IS*S1* inserted itself in the genome. Our inverse PCR sequencing strategy utilized the restriction enzyme, Sau3A1, which cuts throughout the genome, and within IS*S1*, breaking it into two parts (Figure 4C). Ligase, an enzyme that repairs single-stranded discontinuities in double stranded DNA molecules, was then added to the solution. The ligase circularized the DNA. Amongst the DNA containing IS*S1* other circular DNA existed, but with the use of PCR and specific primers only DNA containing IS*S1* were amplified and sequenced. Figure 4C shows the sequencing strategy. Figure 4D shows a map of IS*S1* insertions that we identified through sequencing. The 32 mutants we sequenced were in 31 different genes (all insertions were unique except for *yuiA*) and are spread throughout the genome.

***RTP frequencies*:** Retrotransposition frequency was directly measured with RTP assays (Figure 2). The numbers of colonies formed on the kanamycin plates were used to estimate this frequency as it was compared to the total number of colonies on non-selective plates containing glucose (Novikova et. al 2014). In Figure 5 the different retrotransposition frequencies are shown. The frequencies were calculated by dividing the number of colonies grown on Kan plates by the number of colonies grown on GM17 plates. For our Mutant Set 7 (that was tested here), the frequencies for RTP with and without Cam were about the same. The pRS01 control had a 2.3 fold increase in RTP in comparison to the RIG control. This is due to the pRS01 containing the relaxase gene which stimulates RTP (Figure 1C).

Pooling and high throughput sequencing: The colonies were scooped and gel was run (Figure 5B, left). Then the genomic DNA was sheared; since the DNA was sheared to smaller fragments it ran down to the 100 bp mark (Figure 5B, right). Figure 5C shows a scheme for high throughput sequencing to be used to determine which mutants appear more frequently, to measure how effective they are at RTP. By using P5 and P7 adapters and ISS1 specific primers, high throughput sequencing can be utilized to identify the frequency by which mutants occur. This can therefore show which mutants are stimulatory or inhibitory. The mutant DNA library is being prepared and will be sequenced as a pool.

RTP screening in 96 well format: An alternative method to measuring RTP of our mutants was to screen mutants individually in 96-well plates. Mutants containing the donor plasmid were induced to stimulate RTP. The cultures were spotted to membranes for hybridization (Figure 6A) as well as on GM17 and Kan_{160} plates to select for RTP (Figure 6B). From this assay we identified several "up" and "down" mutants that we are rechecking to verify their effects. We will later sequence these mutants to determine exactly which gene was interrupted in each respective case. In general the radioactive membrane was better at detecting "down" mutants (A), whereas the plating method was superior at identifying "up" mutants (B).

Recombineering Results: Mutated genes can be reconstructed using recombineering. Recombineering allows for the generation of point mutations on the chromosome without use of selection methods such as antibiotics (Britton et., al 2011). Once we identify mutants we will use recombineering methods to make "clean" mutants to further verify their phenotypes. In order for recombineering to be successful in *Lactococcus Lactis* IL1403 the IL1403/pJPOO5 (*recT*) + *nisRnisK* system must be developed. The pJP005 plasmid is a constructed vector containing *recT* gene which has to be transformed into *L. lactis* (van Pijkeren and Britton 2012). *RecT* is a

recombinase and its expression is key in single stranded DNA recombineering since it allows for annealing of complementary DNA (van Pijkeren and Britton 2014). The *nisRnisK* insert is also crucial for *recT* expression; these genes allow for *recT* to be expressed from the nisin promoter. IL1403 does not have the *nisR* and *nisK* genes present in its chromosome. Therefore with strategic cloning, digestion, ligation and transformation this insert can be put in the pJP005 plasmid and then later into IL1403.

We first verified that pJP005 *recT* could be successful in the NZ9000 strain, containing *nisR nisK* genes (Van Pijkeren and Britton 2012) (Figure 7B). For the control, the cells were grown alone. The addition of the single stranded oligo 4195 increased frequency greatly since this oligo has the rpoB mutation that gives rifampicin resistance if recombination occurs in the wild type rpoB gene. The single stranded oligo 4196 had a higher frequency, in comparison to 4195, since it is said to increase efficiency by 10X due to it having the rpoB mutation in addition to 5' phosphate groups that prevent the degradation of DNA (Van Pijkeren et. Al 2012). For recombineering in IL1403, the *nisRnisK* genes need to be cloned into the pJP005 vector since they are essential for the expression of *recT* need to be cloned into the pJP005 vector. The *nisRK* fragment was subcloned into the pGEM intermediate vector and gel purified as a BglII fragment and then ligated to pJP005 BglII linearized vector. Figure 7C shows the nisRK fragment (left gel) and vector and insert purification gel (right). Once the pJP005 *recT* + *nisRK* vector is confirmed, it will be transformed into IL1403, and tested for recombineering efficiency. This system will then be used to create specific mutations in IL1403 to re-test mutant phenotypes obtained in the screen

Conclusion

Overall the purpose of our experiment was to create a mutant library of *L. lactis* using the IS*S1* transposon and test whether the interruption of certain genes affected intron mobility. This mutant library will identify which genes affect retrotransposition of the LtrB intron. This will allow us to understand the relationship of the mobile group II intron and the host cell in the process of retrotransposition. Our mutant library was created using the pG+host plasmid; which we used to introduce the transposon IS*S1* to the genome of *L.lacits* strain IL1403. At the same time we introduced the pLNRK-RIG donor plasmid to study the mobility of
the LtrB group II intron via the RIG cassette.

The results of sequencing the mutants where the IS*S1* transposon inserted itself in the genome of IL1403/pLNRK-RIG, revealed that IS*S1* inserted itself once with an eight base pair-site duplication and inserted itself in different genes amongst the mutants. These results were favorable since we wanted IS*S1* inserted once so that the gene that caused the mutation could be identified easily. In addition, it was evident that IS*S1* was inserted in different genes which will allow the effect of the different genes on LtrB intron mobility to be studied.

Gathering our results and findings, we took our IS*S1* positive RIG positive mutants and did retrotransposition assays. Approximately 1000 mutants have been generated so far, and more mutants will be generated using the same method. *L. lactis* contains approximately 2500 genes, so our goal is to generate 10,000 IS*S1* mutants to ensure coverage of the entire genome four times. With mutants already generated we have begun to identify if IS*S1* insertion sites are unique in the genome. We sequenced 32 mutants and found that the interrupted genes were in diverse parts of the genome. Figure 4D gives a depiction of the *L. lactis* genome and the

respective genes. Amongst them all a few stood out; RecQ an ATP dependent DNA helicase protein, RpoE a DNA-direcred RNA polymerase delta protein, Gpo a glutathione peroxidase protein and CadA a cadium efflux ATPase protein. Further insight into these genes can unveil the behaviors of transposons and mobility pathways in relation to certain genes. Retrotransposition assays should reveal which mutants increase and which mutants decrease retrotransposition. RTP experiments have been done with a set of these mutants and genomic DNA from the RTP is being prepared for high throughput sequencing. We will use a sequencing primer specific to the IS*S1* transposon to identify mutants within each mutant set, and determine if they retrotranspose more or less frequently in comparison to the wild type strain. Those mutants that have an "up" or "down" phenotype will start to give clues as to host factors and pathways that either stimulate retrotransposition or those that may inhibit the process. We have also begun another approach to screen mutants by using a method involving probing of individually induced mutant RTP cultures spotted onto membrane as well as spotted as individual mutants onto selective plates (Kan_{160}). The RTP screening assays appear promising for determining "up" and "down" mutants. These mutations can then be remade using a recombineering technique that we are establishing in the lab. Those genes that are disrupted will be further analyzed.

With methods established in the lab we hope to use our *Lactococcus lactis* mutant library to identify genes that affect retrotransposition of the LtrB intron. In all, this will allow us to better understand retrotransposition in *Lactococcus lactis*. This work will allow us to compare how retroelements relate to hosts in eukaryotic systems, where there are factors both stimulatory and inhibitory. We can therefore compare the arms race between retrotransposons and their hosts in eukaryotic and prokaryotic systems.

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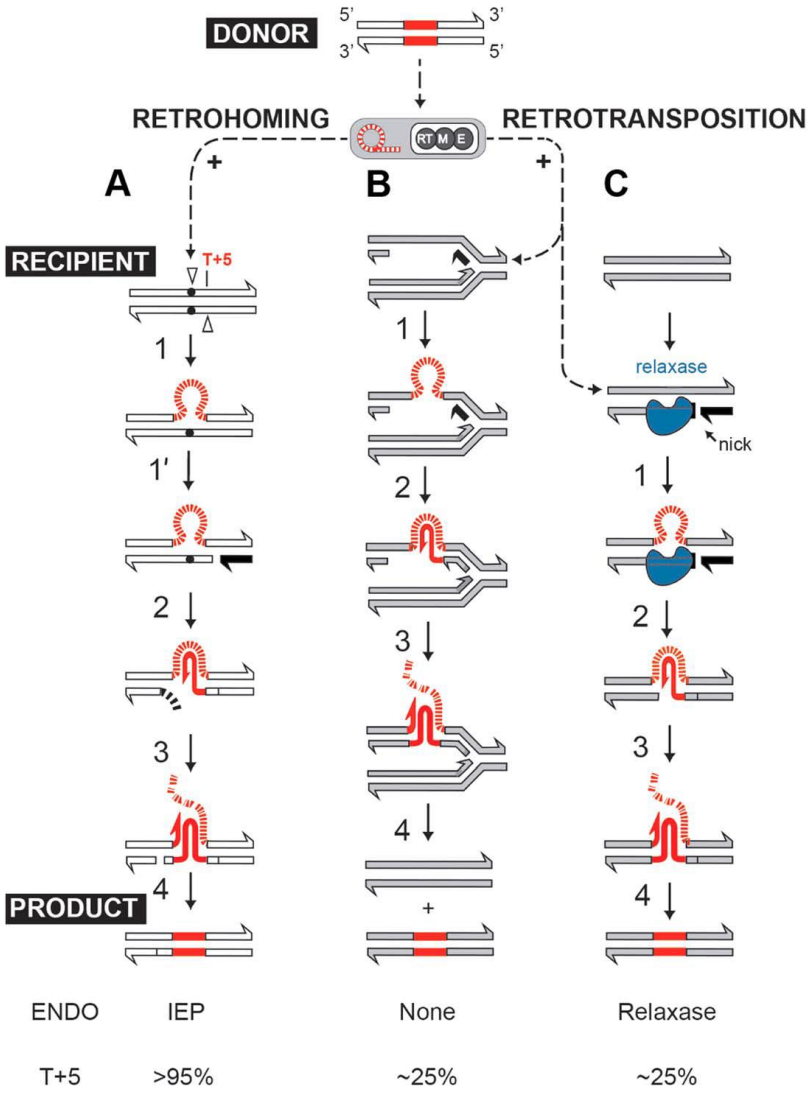


Figure 1. Retromobility pathways. (A) Retrohoming pathways involve an intron moving into its native site. (B) Retrotransposition (RTP) involve introns splicing out forming an intron lariat and moving into ectopic sites of the genome.

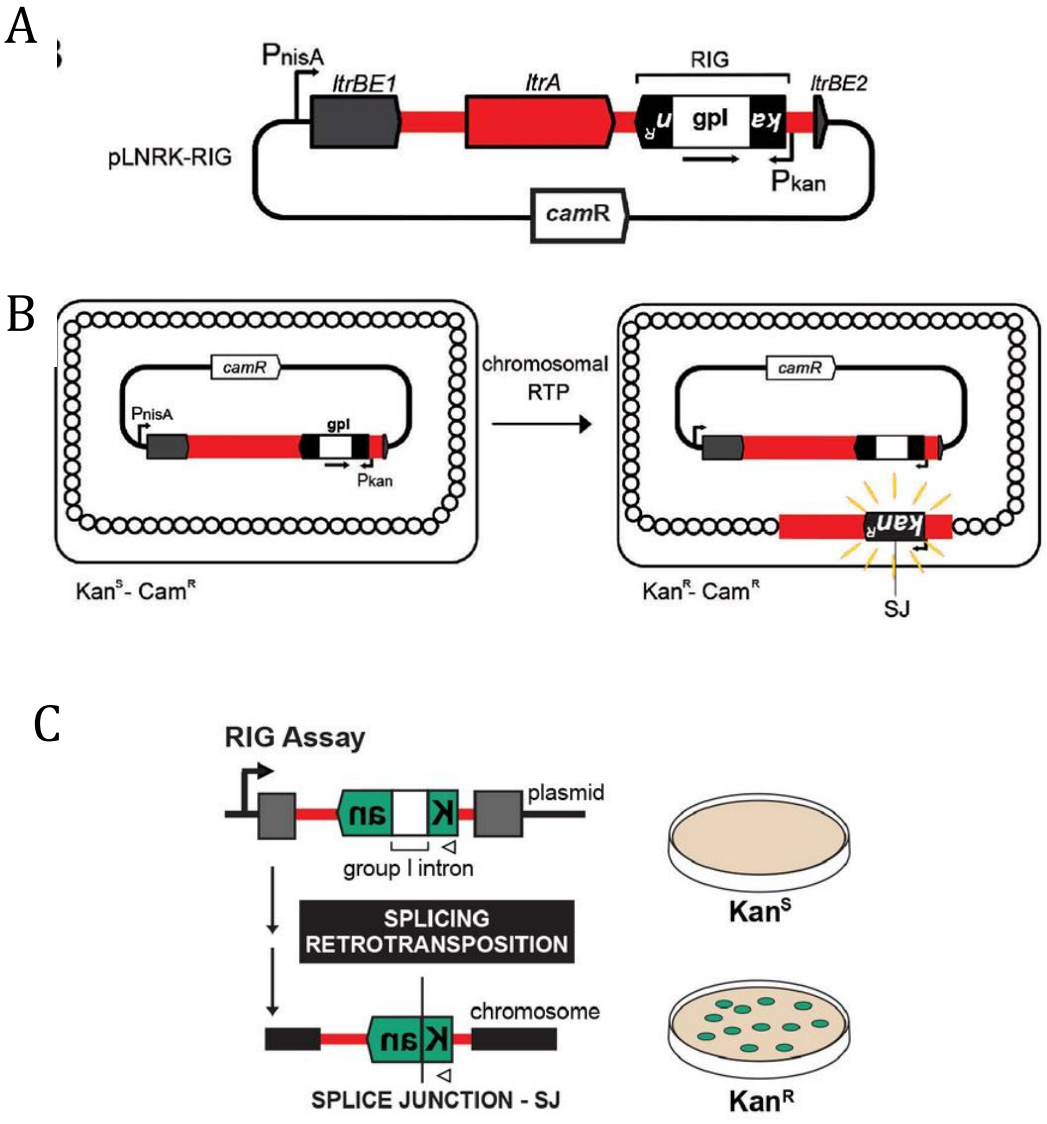
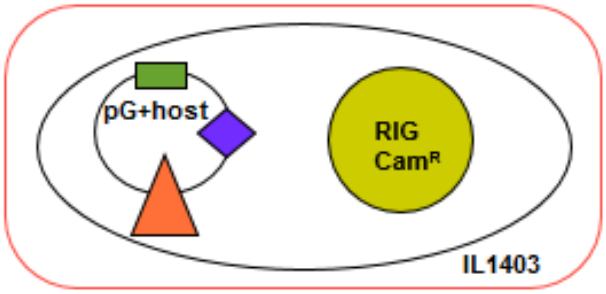


Figure 2: RIG Retrotransposition assay. (A) Engineered Retrotransposition Indicator Gene (RIG) with Kanamycin gene interrupted by group I intron inserted into Ll. ltrB intron carried by intron donor plasmid pLNRK. (B) Left image showing transformation of pLNRK-RIG into Lactococcus lactis host IL1403. Right image showing RTP event occurring causing group I intron splicing out forming splice junction and a Kan^R functional gene inserted into the genome. (C) RIG Assay depicting splicing retrotransposition and basis for assay. Kan^s (Kan sensitivity) showing that an RTP event has not occurred. Kan^R (Kan resistance) showing an RTP has occurred.

A



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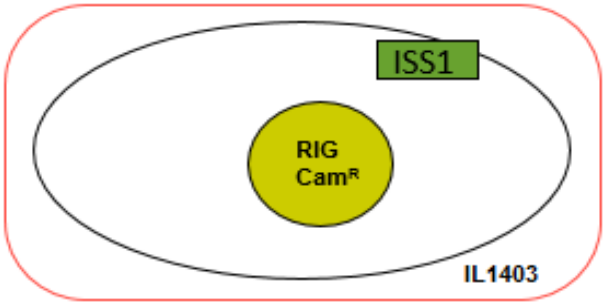

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Figure 3. IS*S1* mutant strategy. (A) Initial transformation of pG+host::IS*S1* and pLNRK-RIG plasmid into *L. lactis* strain IL1403 host. (B) Final step of mutagenesis where ISS1 is inserted once in the genome and the RIG plasmid is maintained in the host cell. (C) Replica plating of mutants to identify those that are Erm sensitive, meaning no pG+host present, and Cam resistant, meaning the RIG plasmid is present.

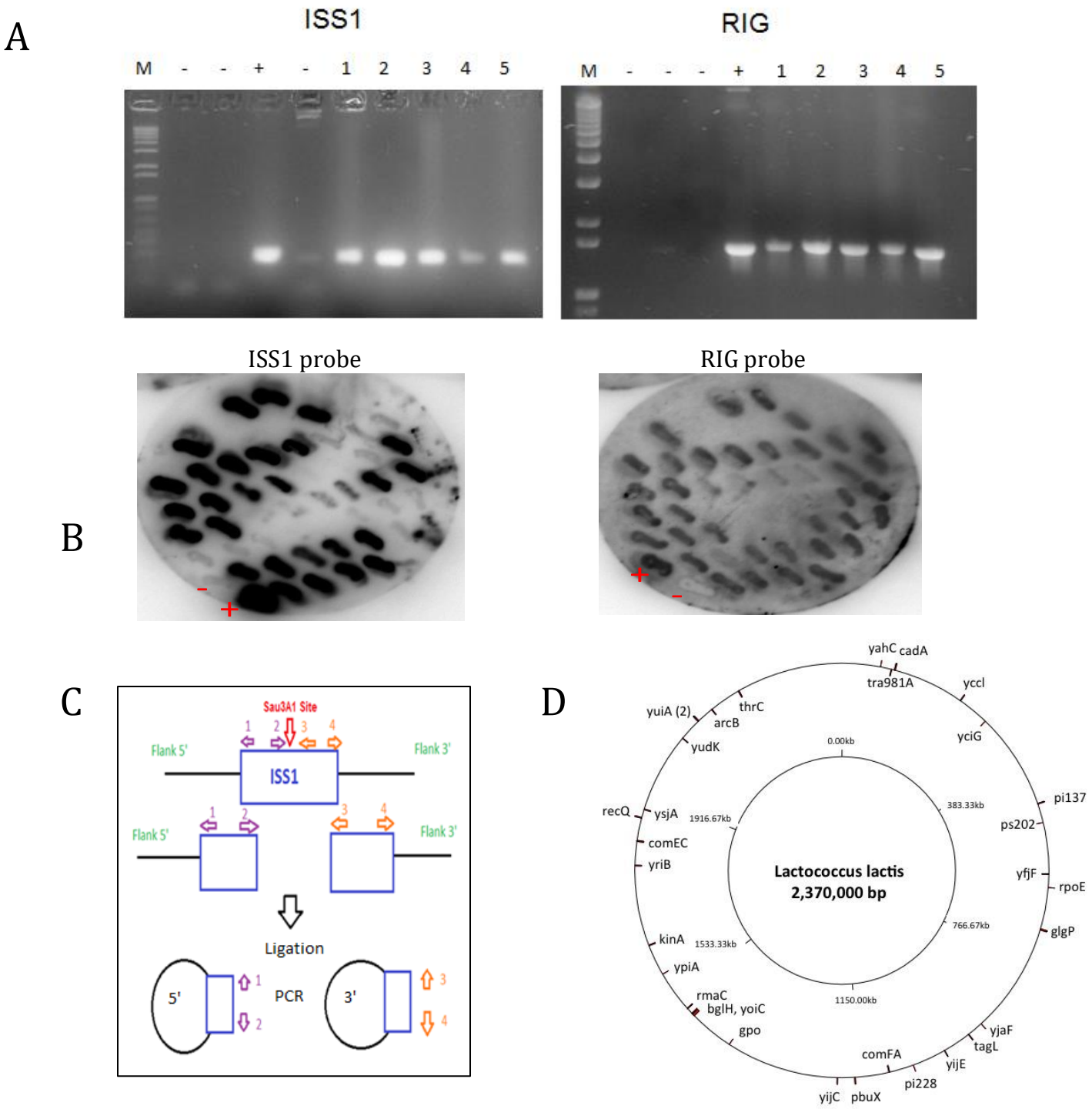


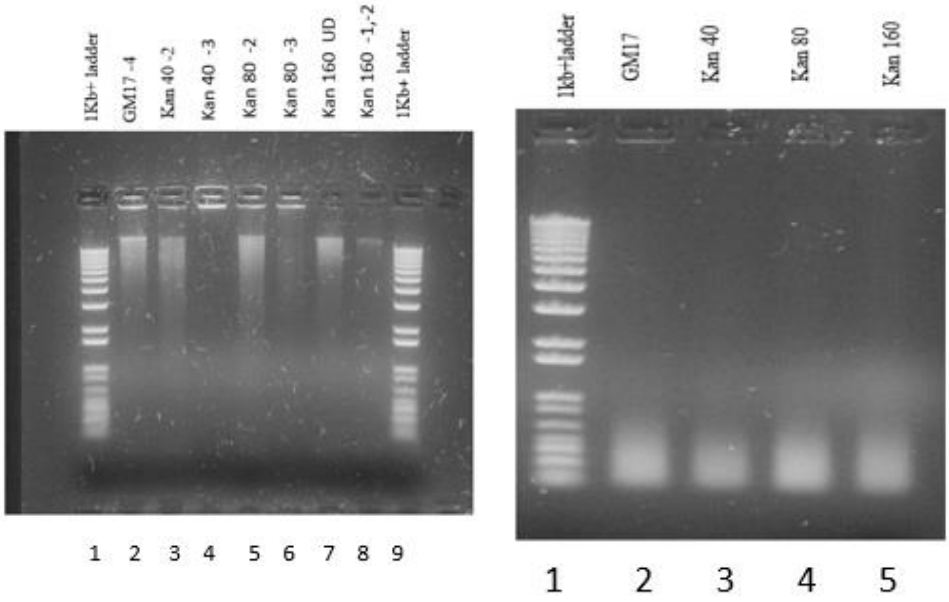
Figure 4: Legend on next page.

Figure 4: IS*S1* verification: (A) PCR's to check the presence of ISS1 and RIG. Image on the left (IS*S1* PCR) ; lane M 1kb+ ladder, lanes "-" signify negative controls (no template DNA, IL1403 genomic DNA, pLNRK-RIG respectively), lane "+" (pG+host::IS*S1*) a positive control, lanes 1-5 various IL1403 mutants. Image on the right (RIG PCR); lane M 1kb+ ladder, lanes "-" signify negative controls (no template DNA, IL1403 genomic DNA, pG+host::IS*S1* respectively), lane "+" positive control pLNRK-RIG, and lanes 1-5 various IL1403 mutants. Bands appear when mutant is positive for either IS*S1* or RIG. (B) IS*S1* and RIG colony hybridization. A dark spot shows a mutant that came out positive for IS*S1* or RIG. And a transparent spot shows a mutant that came out negative for IS*S1* or RIG. (C) IS*S1*sequencing strategy: the use of the Sau3A1 restriction enzyme that cuts frequently in the genome and within the ISS1 gene itself. After Sau3A1 digestion ligase is added to circularize and the use of primers 1,2,3 and 4 will allow for the identification of genes interrupted by IS*S1*. (D) Map of 31 genes IS*S1* interrupted across the *Lactococcus lacti*s genome. All showing unique insertions except for *yuiA*.

A

Mutant Set 7	
Media	Frequency (Kan/GM17)
Kan 40	2.5 x 10-1
Kan 80	3.5 x 10-1
Kan 160	1.7 x 10-4
Control (pLNRK-RIG)	1.5 x 10-5

B



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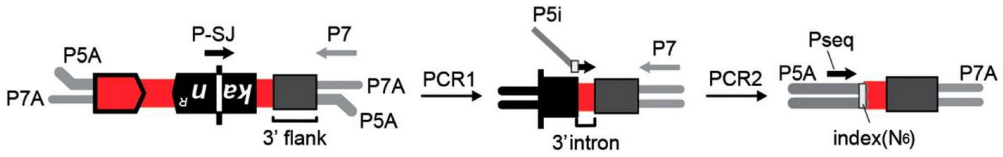
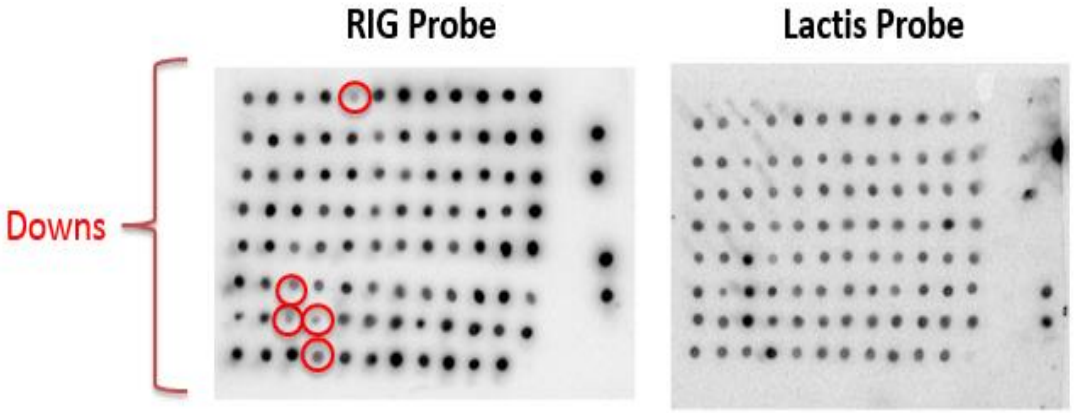


Figure 5. RTP using High Throughput sequencing. (A) RTP frequencies, determined by dividing the number of colonies grown on the kanamycin plates by the number of colonies grown on GM17 plates. Kan 40 having the highest frequency with those mutants showing they can do RTP at some level. Kan 160 having the lowest frequency with those mutants showing they have a greater ability of doing RTP. (B) Genomic DNA of scooped colonies from different dilutions of the 3 Kan concentrations (40, 80, and 160) ran on a gel. Lanes 2, 3, 5 and 7 are unsheared. Lanes 2-5 are sheared DNA (gel on right). (C) High throughput sequencing strategy: use adapters P5 and P7 to allow for the IS*S1* primer to anneal to where the IS*S1* gene is located in the pools of DNA. Those genes that show up frequently are stimulatory to RTP and those that do not show up at all are inhibitory towards RTP.

A



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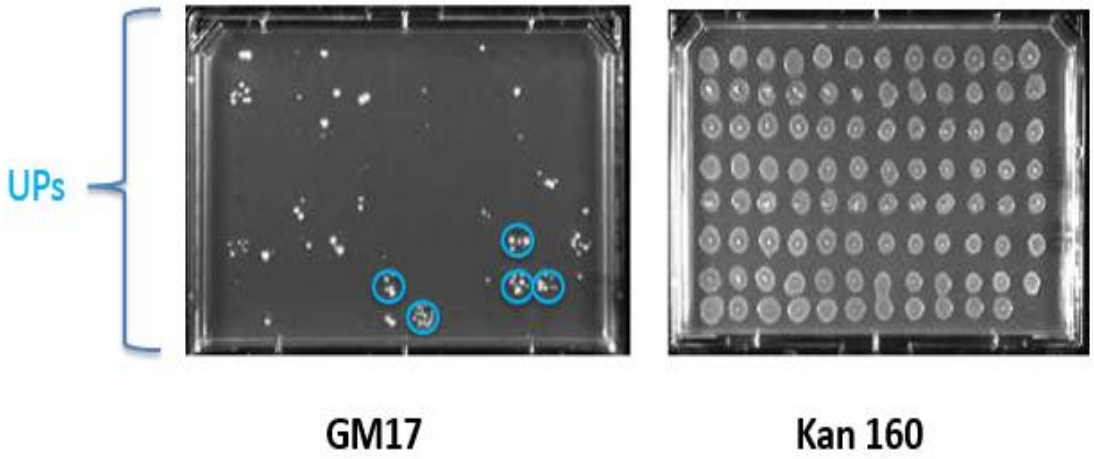
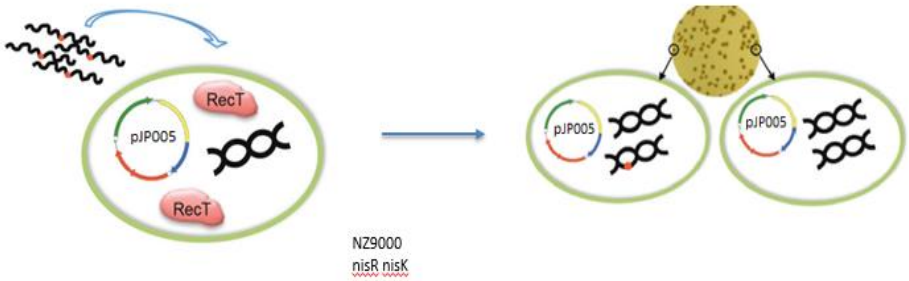


Figure 6. RTP Spot Assay, screening of mutants individually. (A) Biochemical screening: Mutants spotted on a membrane. Lactis probe for general growth and RIG probe detecting RTP events. This method was better at portraying down mutants. (B) Biological screening: Mutants spotted on selective media. This method was better at portraying up mutants.

A



High efficiency recombineering in lactic acid bacteria.
van Pijkeren JP[1], Britton RA.

B

	Frequency (Rif_R/Total Cells)
Control (no oligo)	1.20E-07
4195 (150 ng DNA)	6.80E-03
4196 (150 ng DNA)	2.20E-02

C

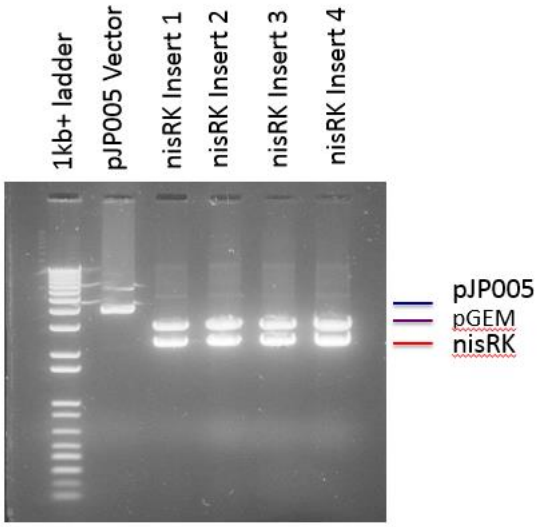


Figure 7. Recombineering in Lactis. (A) Overview of recombineering which starts with transformation of a single stranded oligo containing an engineered mutation. With the help of the *recT* gene the oligo anneals to the chromosome. The results can either be the mutation successfully inserted into the DNA or wild-type DNA. (B) Preliminary test of

recombineering in *L. lactis.* Recombineering frequency was determined b*y* dividing the number of colonies grown on rifampicin media divided by the total cells. Oligo 4195 which contained the *rpoB* gene that causes rifampicin resistance to be expressed showed a lower frequency than oligo 4196 which has the *rpoB* gene along with 5' phosphate groups. No oligo (control) showed the lowest frequency. (C) PCR of isolated pJP005 vector and *nisRnisK* inserts to be cut out and purified to set up nisRnisK + recT pJP005 system needed for recombineering in *L. lactis* strain 1L1403.

Oligos used in this study

IDT 3705	CAG TAA AGT CCT CTA TCA TCT CTG G	Amplification of IS*S1*
IDT 3706	CTA AAT GCA GAA CCA ATA GAG GGC GC	Amplification of IS*S1*
IDT 374	AGC TGA TCA TGT AGT CGA CTA ATG CTT GAA ACC CAG GAC	Amplification of *Kan*
IDT 375	AGC TGA TCA TGT AGT CGA CAG TTG CGG ATG TAC TTC AG	Amplification of *Kan*
IDT 3858	GTT CAT TGA TAT ATC CTC GCT GTC	To sequence from IS*S1* into chromosome 5' end
IDT 3859	CCT GCG TTA CAA TCT AAG CTA TCG TG	To sequence from IS*S1* into chromosome 5' end
IDT 3860	CCG TGA CAA TTA CTC TTG GTT GAC C	To sequence from IS*S1* into chromosome 3' end
IDT 3861	GCC TCA ACC ACG ATT AAG GGC ATG	To sequence from IS*S1* into chromosome 3' end
IDT 4320	AGA TCT TCC CCG GCT TTA GGT ATA GTG	To amplify *nisR nisK* with BglII site - forward primer
IDT 4321	AGA TCT CCG AGC GCA GAA GTG GTC C	To amplify *nisR nisK* with BglII site - reverse primer

Table I: Oligos used in the study. Oligos listed with sequence and function.