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Investigation 6
Plasmid Transfer, Genetic Stability and Nisin Resistance in Lactococci

Abstract

Lactococci are important because they have several applications to the biotech industry. They are used in the dairy industry as cheese starters because they are able to metabolize lactose and produce lactic acid. Bacteriocin genes such as nisin encoded on lactococcal plasmids prevent food from spoiling. The main aim of this investigation was to examine the phenotypic transfer of lactose utilization, proteinase production, and nisin resistance by conjugating two lactococcal strains. Results showed that there were more nisin resistant transconjugants than any other phenotype and this is to be expected since nisin is encoded on a self-transmissible plasmid. It was not uncommon for plasmids of different sizes to be observed in transconjugants due to recombination events facilitated by insertion sequences present on the plasmids. Not all phenotypes matched up with the particular plasmid makeup that would be expected, again because of recombination events between plasmids and also with the chromosomal DNA of the transconjugant.

Introduction

Lactococcal strains have several important industrial applications. In particular the species *Lactococcus lactis* has very simple and well characterized biochemical pathways and is added to milk to start the cheese making process. Starter cultures ferment sugars to produce lactic acid which serves to acidify milk and give cheese its flavor and texture (McAuliffe 2001). Lactococcal enzymes that utilize lactose as an energy source (confer Lac^+ phenotype) are plasmid encoded as are several other essential enzymes. Bacteriophage resistance, protease and peptidase production, bacteriocin production and resistance are also encoded by genes that are present on native lactococcal plasmids (Cotter 2003). Serine proteases (confer Prt^+ phenotype) degrade casein and thus provide the cells with the amino acids they need to grow. Food spoilage caused by pathogenic bacteria is prevented by the bacteriocin nisin that is encoded on several plasmids native to *Lactococcus lactis* (Takala 2002). The cells must also be resistant to nisin if they produce it (Nis^R phenotype). Nisin production and resistance, preptidase production, and lactose utilization all give lactococcus advantages in certain environments and are transferred through conjugation from donor to recipient cells by way of plasmids. Conjugation is dependent on transfer (*tra*) genes and certain self-transmisible plasmids can mobilize other non-transmissible plasmids. Insertion sequences (IS) are present on several lactococcal plasmids and cause recombination events to occur. During conjugation or mobilization, plasmids can recombine so as to produce new plasmids of different sizes and with different genetic makeups. The aim of this investigation was to analyze phenotypic transfer in transconjugants of *Lactococcus lactis* ssp *lactis* by selection of individual markers conferred from donor plasmids.

Materials and Methods

Donor and recipient strains

Donor strain: M189 (Lac^+, Prt^+, Nis^R) Str^S; plasmids 60, 56, 40, 24, 3.7kb
Recipient strain: LM0203 (Lac^-, Prt^-, Nis^S) Str^R; no plasmids

were combined and placed onto a nutrient agar plate to allow for conjugation to occur. The donor strain contained several different sized plasmids. The phenotypes associates with these various sized plasmids are as follows:

- Nis^R on the 60kb and 56kb plasmids;
- Prt^+ on the 56kb plasmid;
- Lac^+ on the 40kb plasmid

The 60kb plasmid encodes *tra* genes and also has insertion sequences so it is therefore self-transmisible and the IS's allow for recombination events to take place. After conjugation, cells were spread onto three different selective media plates in three replicates. The selection plates used were M17L + Sm, FSDA + Sm, and M17G + Sm + Nis; see **Table 1** for an explanation of the phenotypes these selection plates are used to identify. Donor and recipient cells were also separately spread on these selective plates as controls. The number of colonies on each plate was recorded. Ten transconjugants from each of the three selective media plates were selected and streaked for single colonies on their respective medias. These single colonies were then pick and patched onto each of the three selective media plates in order to identify the growth response for the particular transconjugant. The phenotype of the transconjugants was recorded in terms of lactose utilization, nisin resistance, and proteinase production. Within the class each of the different possible phenotypic combinations were chosen and inoculated into M17G broth. The plasmids of these transconjugants were extracted and run on an agarose gel in order to visualize the plasmids present in the transconjugant. See the detailed procedure in the Micro3021 Microbial Genetics Course Kit (Cavicchioli 2005, p. 52-55).

Table 1: Selective media plates used in investigation six and the phenotypes they convey

Selective Media Plate	Biochemical Information Conferred by Growth of Transconjugant
M17L + Sm - Used to determine Lac phenotype	- Contains streptomycin and nisin (Str^S donor and Nis^S recipient will not grow) - Lac^+ cells change this plate from purple to yellow because they utilize lactose and produce lactic acid which causes a pH change in the media. - Lac^- cells can not utilize lactose and the plate remains purple
FSDA + Sm - Used to determine Prt phenotype	This media contains milk - Contains streptomycin so Str^S donor will not grow - Recipient will grow very slowly since it can not access casein in media - Prt^+ cells can break down casein present in the media and the plate turns from an olive color to a creamy color - Prt^- cells do not produce proteinase and thus can not break down casein from the media and the plate remains olive in color

M17G + Sm + Nis - Used to determine nisin sensitivity or resistance phenotype	- Contains streptomycin and nisin (Str^S donor and Nis^S recipient will not grow) - Nis^R cells can grow - Nis^S cells can not grow

Results

After the conjugation mix was plated onto the selective media plates the number of transconjugants was recorded, see **Table 2**. These transconjugant counts show the relative transfer frequency of various phenotypes. The most transconjugants, ~900 colonies, were found on the M17G + Sm + Nis plates followed next by the FSDA + Sm plates with ~500 colonies and the M17L + Sm plates had 15 transconjugants.

Table 2: Initial transconjugant counts on selective media after conjugation

Selective Media	Number of Transconjugants
M17L + Sm	15 (150/ml)
FSDA + Sm	~500 (5000/ml)
M17G + Sm + Nis	~900 (9000/ml)

Table 3: Transfer frequencies of various phenotypes

Phenotype	Percent
$L^+ P^+ Nis^R$	17.8%
$L^+ P^+ Nis^S$	0.70%
$L^+ P^- Nis^R$	13.9%
$L^+ P^- Nis^S$	6.50%
$L^- P^+ Nis^R$	46.1%
$L^- P^+ Nis^S$	3.40%
$L^- P^- Nis^R$	11.6%

After single colonies were pick and patched onto the selective media plates many different phenotypic combinations were observed. The results in **Table 3** show the class frequency of each phenotype as a percentage. There was a higher frequency of the Nis^R phenotype than any other phenotype.

The agarose gel phenotypic profile of our class showed that our results were not adequate to complete the report and so plasmid extraction data (**Figure 1**) and a table of the frequency of transconjugant plasmid profiles for given phenotypes collected throughout the years of this investigation (**Figure 2**) from Jeff Welch were used. In **Figure 1**, lanes two and three are the control donor and recipient strains. All of the plasmids (60, 56, 40, 24, and 3.7kb in size) can be visualized in the donor and there are no plasmids in the recipient. The lanes four through twenty-four show the size of plasmids that are present in cells that exhibit certain phenotypes.

Discussion

Part of the initial transconjugant counts, **Table 2**, and transfer frequency data, **Table 3**, was expected. First, the most growth, ~9000 transconjugants/ml, was observed in the M17G + Nisin + Sm plates and the least growth, ~150 transconjugants/ml, was observed on the M17L + Sm plates. We would expect more nisin resistant colonies (growth on M17G + Nisin + Sm plates) than Lac^+ colonies (growth on M17L + Sm plates) because nisin resistance is encoded on the

60kb plasmid that is self-transmissible and does not depend on any other plasmid to mobilize its transfer and therefore transfers at a greater frequency. Another reason there are more nisin resistant transconjugants than Lac^+ is also because several of the plasmids in the donor strain encoded nisin resistence, both the 56kb and the 60kb plasmids, while only the 40kb plasmid conferred the Lac^+ phenotype. There is an over-representation of Prt^+ transconjugants. The number of colonies per milliliter would be expected to be similar to the Lac^+ since both phenotypes are encoded on a single plasmid that is not self-transmissible. **Table 3** depicting the transfer frequencies of various phenotypes further shows that the Nis^R phenotype is more common than the Lac^+ phenotype and this is because of the same reasons mentioned previously.

Jeff Welch also kept records of phenotype frequencies over several years as shown in **Table 4**. This collection of data has an over-representation of the Nis^S phenotype, in particular the Lac^+ Prt^- Nis^S phenotype is the most frequent of all of the phenotypes. These skewed results can be explained when one considers that this investigation is set up so that each of the possible phenotypes is chosen for plasmid extraction and so the rarer phenotypes appear to be more common than they actually are.

Some of the various phenotypes in **Figure 1** relate to the genotype or plasmid composition that would be expected. For example, lane thirteen has the phenotype Lac^- Prt^+ Nis^R and has two plasmids of sizes 56kb and 60kb. Proteinase is encoded on the 56kb plasmid and nisin resistance is encoded on both the 60kb and 56kb plasmids. The transconjugant did not have the 40kb plasmid that confers lactose utilization and the Lac^- phenotype is fittingly observed. Another transconjugant where the phenotype and plasmid makeup match up is number twenty-three. The phenotype is Lac^+ Prt^- Nis^R and the 56kb plasmid encoding proteinase is missing.

The plasmid extraction gel in **Figure 1** reveals the presence of plasmids that are of different sizes than the ones found in the donor strain. Different sized plasmids with varying genetic composition can be obtained through recombination events among the plasmids. The transconjugant in lane number nine is Lac^+ Prt^+ Nis^S but only has one plasmid that is greater than 60kb. It is likely that the 40kb (Lac^+) plasmid and the part of the 56kb plasmid that confers the Prt^+ phenotype recombined to form this novel sized plasmid that gives this particular phenotype. It is also possible that the *Lac* and/or *Prt* genes recombined with the chromosome of the transconjugant and confer the Lac^+ Prt^+ Nis^S phenotype in that way. Recombination events happen when insertion sequences are present flanking certain genes. These sequences are marked for excision from the plasmid and insertion into either another plasmid or chromosomal DNA. An example of a transconjugant where it is likely that a chromosomal recombination event occurred are lane numbers four and five of **Figure 1**. These transconjugants have 56kb and 60kb plasmids most likely giving them their Prt^+ and Nis^R phenotypes, but there is no 40kb plasmid present that would confer the Lac^+ phenotype that is observed. This data suggests that the *Lac* gene recombined into the chromosomal DNA.

In summary, the nisin resistant transconjugants were more frequent observed because Nis^R is carried on a self-transmissible plasmid and is also present on more than one plasmid while the other phenotypes, Prt^+ and Lac^+, were only carried on one plasmid each. Some phenotypes matched up with their expected genotypes, but there were many that did not. Transconjugants with plasmids of sizes different than those present in the donor strain were observed because recombination events occurred.

References

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