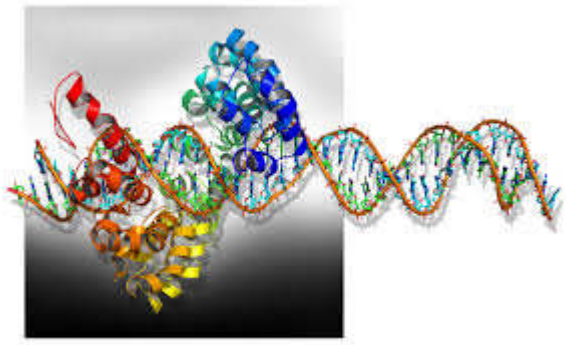
Project number: 5027
Funding source: Teagasc

Date: November, 2014
Project dates: March 2001 – Dec 2007

Genetic Tools for Improvement of Food Cultures



Key external stakeholders:

Starter culture suppliers, fermented dairy food producers, dairy research community.

Practical implications for stakeholders:

- A food-grade cloning system for application in food safety and food quality arena.
- A system where genes for key metabolic and industrial traits can be under-/over-expressed.
- Characterisation of the phage resistance determinants for food-grade improvement of starter cultures.
- Identification of the PyrR gene as a target for the development of bacteriophage resistance strategies in starter cultures.

Main results:

- We over-expressed the plasmid-borne Mg^{2+}/Co^{2+} transporter and investigated it's potential as a marker gene for direct insertional inactivation in lactococci.
- We identified the genetic determinants involved in phage resistance in the conjugative lactococcal plasmid pMRC01.
- We identified the *pyrR* gene as a potential target for improvement of phage resistance properties in starter cultures.

Opportunity / Benefit:

The use of recombinant food cultures requires a food-grade approach to the design of systems for their genetic manipulation. The Genetic Tools for Improvement of Food Cultures program focused on the development of safe and sustainable genetic manipulation systems for various food-grade fermentative and probiotic bacteria. Given that the ultimate aim of research on the biotechnology of food cultures has been to genetically improve strains for food use, much of the focus in recent years has been on the development of self-cloning systems, which rely on genetic elements naturally occurring in the genus. However, the presence of inherent background resistances associated with many of these food-grade markers has placed limitations on their use, something we address in this project. Additionally the wealth of genomic data and information available can be viewed as a positive resource and can be mined for gene-finding strategies.

Collaborating Institutions:

University College Cork
Cork Institute of Technology

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1. Project background:

Food-grade cloning systems totally exclude the use of foreign DNA in molecular manipulation by using only host DNA or the DNA from closely related food bacteria. This food grade cloning approach is essential in the improvement of bacteria as cell factories (producing a range of valuable metabolites impacting on food flavour, human nutrition and health) for food use, especially since the resulting strains may not be considered as "GMOs" as described in EU Directives 219 and 220.

3. The experimental studies:

In developing food-grade genetic tools for lactococcal cultures, we have identified and characterised the Mg2+/Co2+ transport system, CorA, from the lactococcal plasmid pAH90. CorA is the principal Mg^{2+} transport system among the eubacteria. It is also involved in the uptake of Co^{2+}, and hence its presence can be lethal where environmental Co^{2+} concentrations are above a certain threshold. Genetic determinants for CorA activity, comprising the genes *orf18* and *corA*, are found on the lactococcal plasmid, pAH90. Insertionally inactivating the pAH90 *orf18*/*corA* determinants in the presence of Co^{2+} allows cells to grow in concentrations that are otherwise toxic to the cell. We report on the potential of using the lactococcal Mg^{2+} and Co^{2+} transport determinants as a tool for genetic manipulation of lactococci to eliminate background resistances when they are used in conjunction with another marker. In the absence of *corA*, L. lactis NZ9800 is capable of growth in $CoCl_2$ to concentrations of 3-4 mM. In the presence of the *corA* gene and the orf preceding *corA*, *orf18*, these concentrations of $CoCl_2$ are inhibitory. It was found that *corA* is inactive in the absence of *orf18,* a gene encoding a protein of unknown function. Based on the sensitivity phenotype observed, the use of *corA* as a tool for screening clones in *Lactococcus* through insertional inactivation was investigated. Insertional inactivation of the *corA* gene in the presence of $CoCl_2$ allows cells to grow at a concentration of cobalt which would otherwise be lethal to the cell. The lacticin immunity gene was selected for cloning in the *corA/orf18* construct, due to our ability to monitor the phenotype of this gene in addition to the insertional inactivation. We observed that only cells with *ltnI* cloned into the *corA* gene were capable of growth on $CoCl_2$ at a concentration of 2.5 mM. Furthermore, *ltnI* was functional in this construct. This system has the potential to provide an added advantage to current food-grade selectable markers often associated with background resistance by eliminating these resistances and increasing the rate of identification of recombinant clones and the potential of this system has been successfully demonstrated in certain starter lactococci.

An abortive infection mechanism has been previously associated with the fully sequenced, 60.2 kb, conjugative plasmid, pMRC01 (Coakley et al., 1997; Ryan et al., 1996). The mechanism has been shown to target the phage-lytic cycle at a point after phage DNA replication. Plaques formed by the large, prolate-headed phage c2 on L. lactis MG1363 cells harbouring pMRC01 are approximately six-fold smaller than the plaques formed on the MG1363 host itself. The exact location of this Abi mechanism remained unknown. We have identified four genes involved in the Abi mechanism of pMRC01 by a sequential gene-knockout approach that exploits the conjugative nature of the plasmid. In total, four orfs (49, 50, 51, and 52) were linked to the phage resistance phenotype, two of which are homologous to regulatory proteins while the other two encode putative transmembrane proteins.

To exploit the wealth of genomic information generated from starter bacteria (most notably L. lactis IL1403) whole genome microarrays have been used as tools in 'gene-finding' strategies. We exploited DNA microarrays to mine the lactococcal IL1403 genome for genes responsive to infection with the large prolate-headed lactococcal phage c2. This allowed us to identify and characterise potential targets for new anti-bacteriophage strategies. Using these arrays, the global gene expression profile of Lactococcus in response to infection by c2-type and 936-type phages has been evaluated. This approach was used to mine the IL1403 genome for genes that are positively regulated upon infection with phage c2.

4. Main results:

- Insertional inactivation of the plasmid-encoded determinants for Mg^{2+} and Co^{2+} transport, *orf18*/*corA*, provides a tool for screening recombinant clones in *Lactococcus*, based on the observation that overexpression of *orf18*/*corA* results in cell growth inhibition on certain concentrations of $CoCl_2$.

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- We identified the potential of using the lactococcal Mg^{2+}/Co^{2+} transport determinants as a tool for insertional inactivation in lactococci, by eliminating background resistances when used in conjunction with another food-grade marker.
- We overexpressed the plasmid-borne Mg^{2+}/Co^{2+} transporter and investigated its potential as a marker gene for direct insertional inactivation in lactococci and can make the following overall conclusions.
  - (i) CorA requires the preceding Orf18 for Co^{2+} transport activity, and they function as a gene pair, which occurs in at least three other lactococcal strains.
  - (ii) Strains overexpressing the *orf18/corA* determinants cannot grow on $CoCl_2$ concentrations of >2.0 mM, whereas strains in which *orf18* has been replaced with *ltnI* and *corA* has been truncated can grow in concentrations greater than this value.
  - (iii) Since the spontaneous resistance of lactococci expressing *orf18/corA* on solid media after transformation (in the absence of nisin) is less than 1 in 10^9 CFU, the system could be used to identify clones in which the gene has been disrupted.
  - (iv) The system should be applicable to the vast majority of dairy lactococci given the low incidence of plasmid-borne *corA*.
- The potent Abi mechanism associated with the conjugative plasmid pMRC01 involves a four-gene operon, two of which are homologous to regulatory proteins while the other two encode putative transmembrane proteins.
- Induction of this Abi operon resulted in a slower growth rate for the culture.
- Mining the *Lactococcus lactis* IL1403 genome array showed that a number of genes differentially regulated upon phage c2 infection were directly involved in the *de novo* synthesis of pyrimidine nucleotides; *pyrE, pyrF, carB, pyrR, pyrP* and *pyrB*, demonstrating that infecting phage enhance host enzymes involved in nucleotide biosynthesis to generate precursors for phage DNA synthesis.
- It appears that PyrR, the regulator of pyrimidine synthesis in *Lactococcus,* is a limiting factor for phage replication, by limiting the production of pyrimidines. The *pyrR* gene is a potential target for improvement of the phage resistance of food cultures.
- Overexpression of PyrR allows starter acidification rates to be controlled.

5. Opportunity/Benefit:

We have demonstrated the potential of using the lactococcal Mg2+/Co2+ transport genetic determinants as a tool for insertional inactivation in lactococci, by eliminating background resistances when used in conjunction with another food-grade marker. This system provides an added advantage to current food-grade selectable markers often associated with background resistance, by eliminating these resistances and increasing the rate of identification of recombinant clones and the potential of this system has been successfully demonstrated in certain starter lactococci.

The potent Abi mechanism encoded on pMRC01 can be transferred in a food-grade manner into a variety of starter cultures, leading to the concomitant improvement in their phage resistance.

6. Dissemination

Main publications:

- Mills S, Coffey A, Hill C, Fitzgerald GF, McAuliffe O, Ross RP. 2005 Insertional inactivation of determinants for Mg2+ and Co2+ transport as a tool for screening recombinant Lactococcus species clones. Appl Environ Microbiol. 2005 Aug;71(8):4897-901.
- Mills S, Coffey A, McAuliffe OE, Meijer WC, Hafkamp B, Ross RP. 2007 Efficient method for generation of bacteriophage insensitive mutants of Streptococcus thermophilus yoghurt and mozzarella strains.
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- Trotter, M., McAuliffe, O., Callanan, M., Edwards, R., Fitzgerald, G. F., Coffey, A. Ross, R. P. 2006. Genome analysis of the obligately lytic phage 4268 of Lactococcus lactis provides insight into its adaptable nature. Gene. 366 (1): 189-199.
- McAuliffe, O., Ross, R. P. and Fitzgerald, G. F. 2006. The new phage biology: from genomics to applications. In Bacteriophage: Genetics and Molecular Biology, Horizon Scientific Press.

7. Compiled by: Sheila Morgan

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