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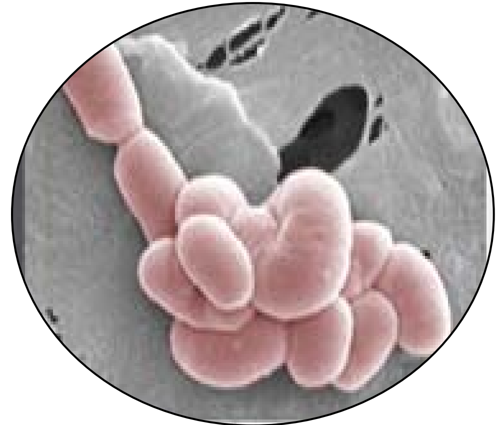
Phage-insensitive cultures for the production of fermented and probiotic foods



Key external stakeholders:

Commercial culture suppliers; fermented dairy food producers; wider dairy industry; lactic acid bacteria and phage research communities.

Practical implications for stakeholders:

Bacteriophages are the primary cause of fermentation failure in the fermented dairy foods industry. Lysis of the starter culture can delay or even halt the milk fermentation process leading to low quality products, or even discarding of the milk. The destructive potential of these agents is exaggerated in modern processes which employ cultures on a more or less continuous basis and where huge numbers of starter cells are required to process large volumes of milk to cheese. The economic impact of such attacks can be significant, particularly in a commodity product such as cheese where profit margins are very tight.

The main outcomes generated from this project are:

- Food-grade strategies have been developed to improve commercial starter cultures with respect to bacteriophage resistance.
- Improved cultures have been transferred to industry where they have replaced bacteriophage-sensitive strains, thus improving the efficiency, reliability and longevity of starter cultures.

Main results:

- The molecular mechanisms underpinning phage-host interactions were characterized. The host response is strongly targeted to the cell wall, suggesting that the phage presence is sensed as an extracytoplasmic stress affecting membrane integrity.
- Phages infecting commercial probiotic cultures were isolated and characterized.
- Classical food-grade approaches and novel mobilizable plasmids were used to improve the phage-resistance phenotype of commercial starters, some of which have been transferred to industry.

Opportunity / Benefit:

There is an ongoing opportunity for other starter culture and dairy companies to benefit from the capabilities developed within this project though sponsored contract research or service provision. Expressions of interest from relevant companies are welcome.

Collaborating Institutions:

University College Cork.

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1. Project background:

The manufacture of fermented food products has a major impact on the Irish economy. While Cheddar cheese still accounts for the bulk of this market, there has been considerable diversification in recent years to other established products, such as yoghurt, Mozzarella, Swiss and Dutch type cheeses as well as the development of new probiotic products. The manufacturing processes for these foods is critically dependent on the consistent performance of starter cultures such as *Lactococcus lactis*, *Streptococcus thermophilus* and *Lactobacillus* spp. Bacteriophages (viruses which kill bacteria) represent the most significant cause of starter culture inhibition in commercial fermented food production. The destructive potential of these agents is exaggerated in modern processes that intensively employ cultures and where huge numbers of starter cells are required to process large volumes of milk. The commercial consequences of phage infection include disruption of production schedules, reduction in product quality, reduction in commercial value and, in the most severe cases, abandonment of production. In the past, a variety of phage resistant cheese-making strains, which were suitable for use in industry, have been generated in the laboratory. While this has been very helpful to industry, it has been observed that phages, over time, evolve and adapt to circumvent these natural resistance mechanisms. This is a problem when strains are relied upon for extended cheese manufacturing schedules and it calls for more sophisticated approaches to tailoring the phage resistance mechanism to combat a particular phage type. This project therefore exploited the extensive knowledge base built up over the past 20 years, leading to better protection of bacterial cultures in the long term. The research also provided a greater understanding of molecular mechanisms involved in the phage-host interaction. In addition, the project addressed a major potential problem i.e. phage against probiotic cultures.

2. Questions addressed by the project:

- Can methods developed at TFRC-Moorepark be used to improve the phage resistance properties of commercially-used dairy starters?
- Can phage be isolated against currently used probiotic cultures?
- Do artisanal dairy cultures harbour plasmids with novel phage resistance mechanisms?
- Can these plasmids be used to improve the phage resistance properties of commercially strains?
- Does the presence of large plasmids in dairy starters affect their performance?
- Does a bacterial cell undergoing phage attack mount a defense strategy?
- Can knowledge of this strategy be used to develop strategic anti-phage solutions?

3. The experimental studies:

One of the main objectives of this project was to develop the capability to generate phage-resistant variants of commercial cultures with a view to supplying these cultures to industry. Methods previously developed by our group based on classical approaches for the isolation of bacteriophage-insensitive mutants, were used to create variants of commercial strains of *Lactococcus lactis* and *Streptococcus thermophilus*. Newly-generated phage-insensitive variants were tested for their technological performance and their genetic fingerprint and transferred to industry once these criteria had been met. Phage–resistant variants of commercial starters were also generated using food-grade conjugation methods through the transfer of mobilizable plasmids encoding phage-resistance mechanisms into phage-sensitive starters. Transconjugants were tested for their ability to withstand phage infection.

Bacteriophages against probiotic cultures were isolated from sewage using methods developed in this project. Basically, a pre-enrichment method was developed to increase the titre of phages in the collected sewage samples and activity was tested against a range of probiotic strains including *Lactobacillus paracasei*, *Lactobacillus rhamnosus* and several strains of *Bifidobacteria*. Bacteriophages against starter and probiotic bacteria and plasmids of interest were also characterized genetically using Sanger and 454 DNA sequencing. The resultant sequence information was assembled and annotated using the GAMOLA bioinformatics suite.

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To study the impact of plasmids on starter culture performance, the degree of autolysis of strains was evaluated by monitoring the levels of lactate dehydrogenase (LDH) released after growth. Also, flow cytometric analysis was used to qualitatively assess the autolytic phenotype.

DNA microarrays were used to evaluate the impact of phage infection on the lactococcal starter *L. lactis* IL1403. Significant time was taken to develop methods to analyse gene expression using DNA mciroarrays. Changes in the level of gene expression of each annotated open reading frame of IL1403 to infection with lactococcal phages c2 was monitored and confirmed with RT-PCR.

4. Main results:

The main findings of the project were as follows:

- A number of commercial cultures were improved with respect to their phage resistance properties using methods previously established at Moorepark. Some of these cultures have been transferred to commercial starter companies, who have produced the cultures for sale to their clients.
- A novel phage lytic for the probiotic bacterium *Lactobacillus paracasei* 338 was isolated from sewage using a newly developed enrichment method and was characterized at the structural and genetic level. Electron microscopy studies revealed that φLb338-1 is a member of the *Myoviridae* family, with an isometric head, a medium-sized contractile tail, and a complex base plate. Genome sequencing revealed a 142 kb genome with 199 open reading frames. Phage Lb338-1 was found to be a member of the broad-host-range SPO1-like group of phages.
- Analysis of lactococcal strains isolated from artisanal cheeses revealed a wealth of technologically important traits encoded on mobilizable plasmids. Plasmid sequencing revealed the presence of a number of novel restriction/modification (r/m) phage resistance systems, which were characterized as Type I r/m systems due to the presence of HsdS subunits.
- Plasmids encoding novel r/m systems were mobilized from *L. lactis* subsp. *cremoris* DPC3758 to lactococcal starters and shown to improve the phage resistance properties of these strains. These plasmids are currently being used to improve the phage resistance properties of commercial strains.
- The impact of the presence of plasmids on starter performance was evaluated. Starters containing pMRC01 exhibited lower specific growth rates and higher generation times compared to the parental strains, but the presence of pMRC01 did not significantly affect the acidification capacity of strains. Analysis by flow cytometry following live/dead™ staining confirmed an increased cell permeability and autolysis, which did not affect the acidification capacity of the starter
- A study of the interaction between phage and hosts using DNA microarray technology revealed that the genetic response of *L. lactis* IL1403 to infection by phage c2 involves a four-strand approach. Transcriptome analysis revealed the regulation of genes involved in membrane stress, D-alanylation of the cell wall, maintenance of the proton motive force and energy conservation.

5. Opportunity/Benefit:

The expertise at Teagasc to characterise and improve cultures with respect to phage resistance and other important technological traits has been transferred to two international starter culture companies. Indeed, a major component of the R & D activities of both of these companies is located at Moorepark. There is an ongoing opportunity for other starter and dairy companies to benefit from these capabilities though sponsored contract research or service provision.

6. Dissemination:

Main publications:

Fallico, V., R. P. Ross, G. F. Fitzgerald and O. McAuliffe. 2011. Genetic response to bacteriophage infection in *Lactococcus lactis* reveals a four-strand approach involving induction of membrane stress proteins, D-alanylation of the cell wall, maintenance of proton motive force and energy conservation. *J. Virol.* 85: 12032-12042.

Fallico, V., O. McAuliffe, G. F. Fitzgerald, and R. P. Ross. 2011. Plasmids of raw milk cheese isolate *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* DPC3901 suggests a plant-based origin for the strain. *Appl. Environ. Microbiol.* 77: 6451-6462.

Alemayehu, D., R. P. Ross, O. O'Sullivan, A. Coffey, C. Stanton, G. F. Fitzgerald and O. McAuliffe. 2009. Genome of a virulent bacteriophage Lb338-1 that lyses the probiotic *Lactobacillus paracasei* cheese strain.

Gene 448: 29-39.

Popular publications:

McAuliffe, O., R. P. Ross and G. F. Fitzgerald. 2007. The new phage biology: from genomics to applications. *In* Bacteriophage: Genetics and Molecular Biology. Eds. S. McGrath and D. van Sinderen. Horizon Scientific Press, UK.

Culture wars on phage attack. *Moorepark News*, Winter 2008.

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