View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by T-Stór

Development and Application of Strategies to Generate Bacteriophage Resistant Strains for Use in Milk Fermentation Processes

(Development of Phage Resistant Starter Strains)

Armis No. 4206

Project Team:
R.P. Ross and G.F. Fitzgerald (Leaders)
A. Coffey
M. Coakley
D. O'Sullivan

The Dairy Products Research Centre
Moorepark, Fermoy, Co. Cork.

Teagasc acknowledges with gratitude grant aid under the Food Sub- Programme (Sub-Measure 3 (ii) - Institutional R&D) of the Operational Programme for Industrial Development. The Programme is managed by the Department of Agriculture and Food supported by EU and national funds.

ISBN 1 901138 32 1

DPRC No.5

Summary and Conclusions

Phage (bacterial virus or bacteriophage) infection of starter cultures poses a major problem in the manufacture of a range of fermented milk products of considerable importance to the Irish economy (Cheddar cheese, yoghurt, lactic butter) and can result in significant monetary losses. The phage retard or, in the case of severe infection, eliminate the activity of the starter resulting in low grade product, and in extreme cases, total product loss. Since it is extremely difficult to physically exclude phage from the manufacturing environment, specific strategies must be employed to protect the cultures so that they can perform the fermentation process in a rapid, predictable manner, yielding product of the highest quality. Molecular genetic techniques offer a rational solution to the problem whereby phage-sensitive lactococcal starters can be given a range of genetic defences to enable them to withstand the onslaught of phage attack.

At Moorepark, three different natural phage resistance systems which target different stages of the life cycle of the virus have been identified in specific lactococcal strains. This followed an analysis of a variety of lactococci from the Moorepark culture collection, currently composed of over 5,000 strains of lactic acid bacteria. Methodologies were then established for transferring the genetic determinants for these phage resistance systems to cheese-making starter strains. Following this, phage resistance determinants were transferred by a natural process, known as bacterial conjugation, to a variety of starter strains conferring increased phage resistance on these strains.

Hence the objectives of this project were firstly, the identification of natural phage resistance systems for exploitation, secondly, the development of methodologies to utilise these systems to improve the bacteriophage resistance of starter strains for use in milk fermentation processes, and thirdly, the actual application of these methodologies to improving starter strains.

The main conclusions were as follows:

- Three new natural plasmid (DNA)-associated bacteriophage resistance systems were identified at Moorepark. The detailed genetic makeup of the phage resistance plasmid (pMRC01) was elucidated.
- Bacteriophages currently evolving in the industrial cheese-making environment were monitored to facilitate the judicious choice of phage resistance systems for use in commercial starter cultures which can more effectively target the documented problematic phage types.
- Two highly virulent phages targeting important cheese starters were identified in the industrial cheese-making environment.
- A reliable food-grade method to facilitate the transfer of phage resistance systems to cheese-making starter strains was developed. This is based on bacteriocin immunity-linked phage resistance.
- Phage resistant cheese starter cultures were developed through natural selection and by molecular manipulation using phage resistance plasmids. The phage resistance plasmid pMRC01 was introduced to 31 cheese starter strains.

Commercial impacts

- All phage resistant starter strains resulting from the strain improvement research were evaluated under stringent laboratory conditions. Selected strains were then evaluated in pilot-scale Cheddar cheese trials and were subsequently validated in commercial cheese plants.

Research and Results

New problematic phages from commercial cheese-making environments

A virulent phage, designated phage 4268 was identified as a potent inhibitor of one of the principal strains used for Cheddar cheese-making in Ireland (designated DPC4268, see *Fig. 1a*). This phage was isolated from industrial cheese whey samples obtained from seven production runs of DPC4268. Due to the importance of this starter and the lack of previous reports of a phage against it, effort was devoted to its characterisation. The plaque diameter of phage 4268 on DPC4268 was 0.6mm, the burst size was between 42 and 50 pfu and the latent period was estimated at 50 min. Electron microscopy studies indicated that phage 4268 is small isometric-headed, having a head diameter of 62 nm, a tail length of 274 nm, a tail width of 11.2 nm, and a tail fibre of 96 nm. A base plate and distinctive tail crossbars are also evident. Phage 4268 DNA was subjected to restriction analysis and the size of its genome was estimated to be 41 kb. Comparison of restriction patterns resulting from heated (70°C) and unheated digests confirmed that the phage is cohesive ended. Preliminary DNA hybridisation analysis indicated that phage 4268 was unrelated to either P008 type or P335 type phages, the two most common phage types encountered in Ireland. In addition, the phage was also genetically unrelated to the morphologically similar P087 type. Further DNA hybridisation analysis indicated that phage 4268 did not originate from a temperate phage. The phage itself was subsequently used to generate a phage resistant mutant of the cheese starter DPC4268.

A second virulent phage, designated phage 4932 (Fig. 1b) was identified more recently as a potentially serious inhibitor of another important Cheddar cheese starter DPC4932. This lytic phage was isolated from whey samples from industry and studied in detail. The plaque diameter of phage 4932 on DPC4932 was unusually large at 5.0 mm. Electron microscopy studies indicated that phage 4932 is prolate-headed, having head dimensions of 45 x 63 nm,

a tail length of 120 nm, and a tail width of 12 nm. Distinctive tail crossbars are also evident. As part of one of the tasks in the project, the introduction of pMCR01 to strain DPC4932 reduced the plaque size of this prolate phage significantly from 5 mm to 0.5 mm.

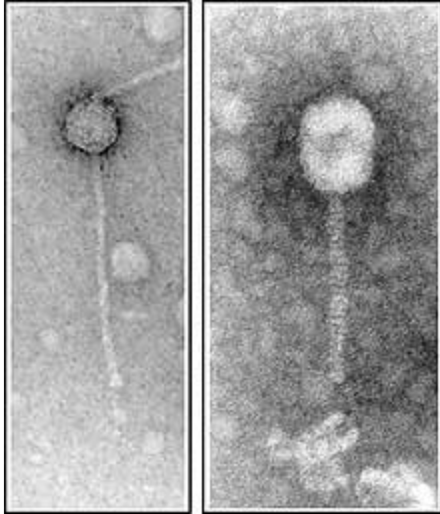


Fig. 1. Electron micrographs of problematic industrial phage isolated from Cheddar cheese plants. (A) Small isometric-headed phage 4268 which attacks the starter DPC4268 and (B) Prolate-headed phage 4932 which attacks the starter DPC4932.

Identification of three natural phage resistant systems

The plasmid pMRC01 was identified as a potentially useful phage resistance plasmid.

It was found to be multi-purpose plasmid encoding both phage resistance and production of (and immunity to) lacticin 3147, a novel broad spectrum bacteriocin. The genetic linkage of phage resistance and bacteriocin determinants on such plasmids in lactococcal starters may offer a very efficient approach towards starter strain development since strains improved with respect to their phage resistance can easily be selected based on their immunity to bacteriocin. Phenotypic characterisation of strains containing the pMRC01 plasmid have demonstrated that it mediates abortion of phage infection at a point in the phage cycle after phage DNA replication. The nucleotide sequence of the plasmid was elucidated in full and the abortive infection system was shown to be distinct from existing abortive infection systems on the basis of comparison with nucleotide sequence databases.

Studies on another phage resistance plasmid, pAH90 were also undertaken. This plasmid, designated pAH90 harbours phage resistance mechanisms which can complement that present on pMRC01, was studied in detail at the molecular level. This second plasmid encodes phage adsorption inhibition and restriction/modification, mechanisms which target different points in the phage life cycle than the abortive infection mechanism encoded by pMRC01. pAH90 is, in essence, a co-integrate of two smaller plasmids, pAH82 and pAH33. The nucleotide sequence of one sub-component of pAH90, namely pAH33, was elucidated and the co-integration site necessary for the formation of pAH90 was identified.

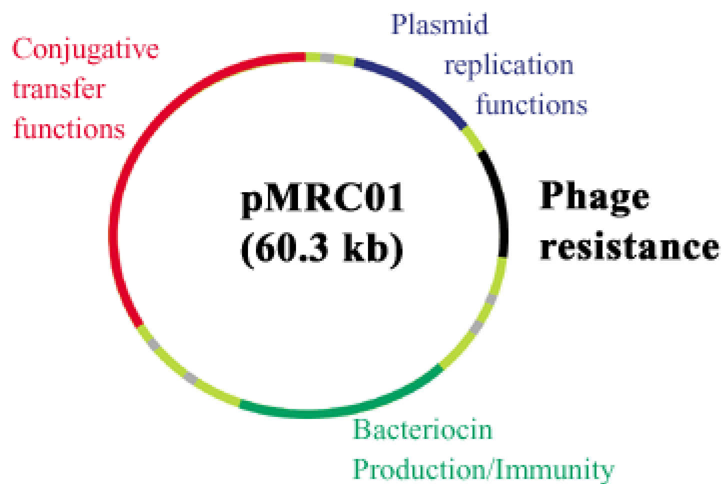


Fig. 2. Circular representation of the phage resistance plasmid pMRC01. This plasmid has been characterised in great detail and has been used extensively in starter strain construction.

Development of a strategy to transfer phage resistance determinants to cheese starters

The pMRC01 plasmid was evaluated for its potential usefulness for improving the phage resistance properties of starters. The plasmid was firstly introduced into a food-grade donor strain namely *L. lactis* MG1363. A plasmid transfer protocol was then developed and used to attempt to transfer it from this donor strain into selected lactococcal strains similar to those currently used for Cheddar cheese and lactic butter manufacture. These transfers were performed in a Food Grade manner via conjugations after which the newly modified strains were selected based on their immunity to the bacteriocin.

Preliminary characterisation demonstrated that many of the resultant starters had improved phage resistance (Fig. 3) and produced bacteriocin. Moreover, these modified strains performed well with respect to commercially important traits such as acid production and/or diacetyl production.

Transfer of phage resistance plasmid to a variety of Cheddar cheese starters

Owing to the success of the plasmid delivery protocol, the plasmid pMRC01 was subsequently successfully introduced into a variety of Cheddar cheese strains. Acquisition of the plasmid by these strains was demonstrated by using each of the transconjugants as donors in second-round conjugations. In addition, specific PCR primers were also designed and used to confirm acquisition of the plasmid by cheese starter strains. Electroporation was also adapted as a useful alternative approach for introducing the phage resistance plasmid to specific strains. In all, 50 target recipient strains for pMRC01 were chosen. The plasmid was successfully introduced to 31 of these *(Table 1)*. The panel of recipient strains included a number of autolytic cheese starters.

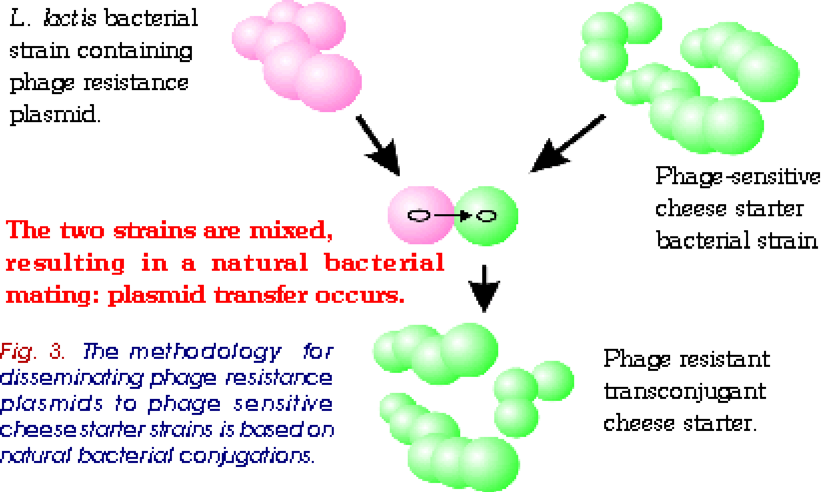


Fig. 3. The methodology for disseminating phage resistance plasmids to phage sensitive cheese starter strains is based on natural bacterial conjugations.

Autolytic strains are notoriously phage sensitive and thus difficult to use in industry, nevertheless, the autolytic property can have a major impact on cheese flavour in that it causes the release of greater amounts of intracellular degradative enzymes into the cheese curd than would occur in non-autolytic strains. The plasmid was successfully introduced into four autolytic cheese starters.

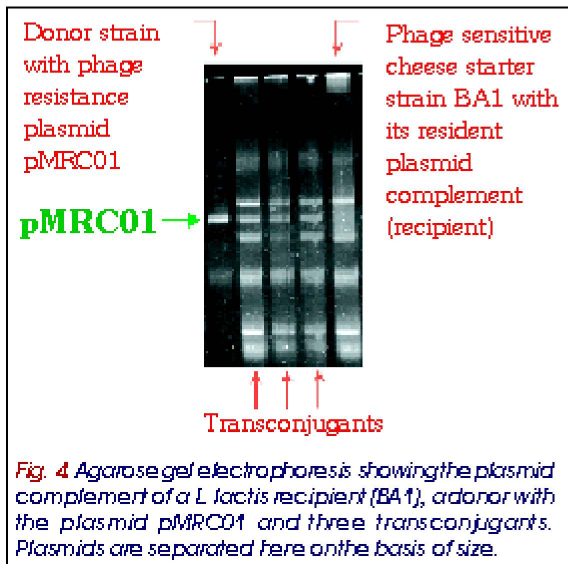


Fig. 4 Agarose gel electrophoresis showing the plasmid complement of a L lactis recipient (BA1), a donor with the plasmid pMRC01 and three transconjugants. Plasmids are separated here on the basis of size.

Fig. 5. Bacteriophage plaque assays on a commercial lactococcalCheddar cheese starter DPC4932. The left plate shows phage plaques (diam. 5mm) on a lawn of DPC4932 cells. In the case of the plate on the right, pMRC01 has been introduced to DPC4932 with the result that phage plaquing ability is drastically reduced to the extent that plaques are pinpoint and barely visible. The phage used in this assay is phage 4932, a highly virulent prolate phage identified in a number of commercial cheese plants.

In many cases it was necessary to adapt or modify the conjugation protocol to suit individual strains. Overall, the conjugation experiments provided an important insight into the transferability and acceptability of large conjugative phage resistance plasmids in cheese starters. Not all strains accept the plasmid for various reasons, and some of the strains which did accept the plasmid were not protected against all the phages available. In such cases the development of bacteriophage insensitivie mutants was useful to protect the starters.

Table 1. Lactococcus lactis cheese starter strains to which the phage resistance plasmid pMRC01 was introduced.

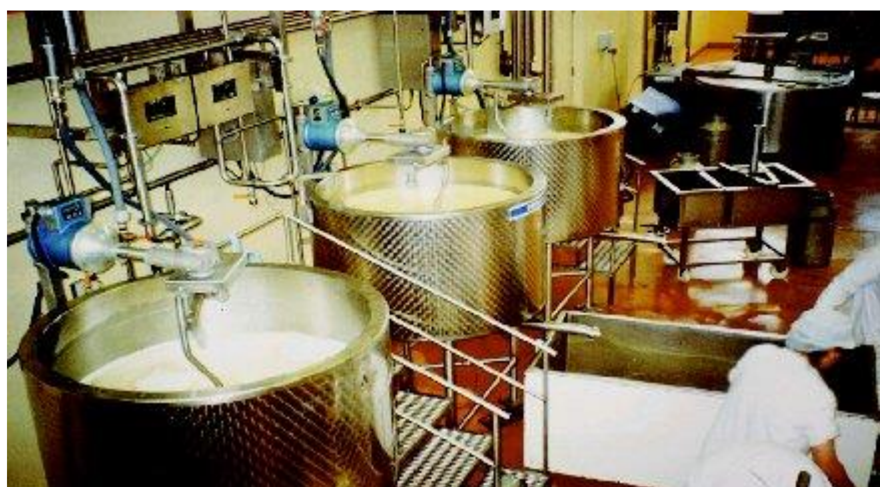
L. lactis	**BA1**	***L. lactis***	**BA2**
"	SK1	"	US3
"	AM1	"	C25
"	Z8	"	KHNZ
"	HT2	"	HT7
"	HT8	"	DPC4268
"	DPC4272	"	DPC4273
"	DPC4274	"	HP
"	NCD0712	"	ML8
"	077	"	007
"	MG1363	"	MG1614
"	DPC3290	"	DPC4932

"	DPC4933	"	DPC4934
"	DPC4935	"	DPC4936
"	DPC4937	"	DPC3343
"	DPC220		

Validation of phage resistant strains at pilot and commercial scale

Lactococcus lactis DPC4830, a bacteriophage resistant derivative of the widely used Cheddar cheese strain DPC4268 which was developed during this project was evaluated in a pilot-scale cheese trial at Moorepark.

Pilot-scale Cheddar cheese manufacture with phage resistant strains.



In the trial, the phage resistant strain performed well under manufacturing conditions and resulting 6-month-old cheeses received flavour scores equal to, or better than, cheeses made with the parent strain. This strain was subsequently validated in a commercial cheese plant where it yielded a product of exceptional quality. In addition, subsequently, the phage resistant derivative of the strain DPC4932 (DPC5020) was validated in two commercial cheese plants.

For further information, please contact:

Dr. R. Paul Ross - Dr. Aidan Coffey

Publications

Ross, R.P., Fitzgerald, G.F. and Coffey, A.G. (1995). Increasing the phage resistance of starter cultures. In: *4th Cheese Symposium,* Teagasc, Fermoy, Co.Cork.. 110-118.

Coakley, M., Fitzgerald, G.F. and Ross, R.P. (1995). Bacteriocin-linked phage resistance for the improvement of lactococcal starter cultures. *Irish Journal of Agricultural and Food Research*. 34 217 (Abstract).

Coakley, M., Fitzgerald, G.F. and Ross, R.P. (1996). Evaluation of the phage resistance plasmid pMRC01 for the improvement of lactococcal starters. *Proceedings of the 5th Symposium on Lactic Acid Bacteria, Genetics, Metabolism and Applications*, Veldhoven, The Netherlands. Abstract F18. © 1996 Kluwer Academic Publishers.

Coakley, M. (1996). Application and Evaluation of the phage resistance plasmid pMRC01 for the improvement of lactococcal staters. *MSc.Thesis*, National University of Ireland.

Coakley, M., Fitzgerald, G.F. and Ross, R.P. (1997). Evaluation of the phage resistance/bacteriocin plasmid pMRC01 for the improvement of dairy starters. *Applied and Environmental Microbiology*. 63 1434-1440.

Coffey, A.G., Fitzgerald, G.F. and Ross, R.P. (1997). Molecular biology of starter cultures: has it paid off? In: *5th Cheese Symposium*,Teagasc, Fermoy, Co.Cork. 42-52.

Coffey, A.G., Fitzgerald, G.F. and Ross, R.P. (1997). Biotechnology of Cheese starter cultures. *The Biochemist*. August 1997.

Coffey, A.G., Coakley, M., McGarry, A., Fitzgerald, G.F. and Ross, R.P. (1997). Increasing the phage resistance of cheese starters: a case study with *Lactococcus lactis* DPC4268. *Letters in Applied Microbiology*. 26 51-55.

O'Sullivan, D., Coffey, A.G., Fitzgerald, G.F. and Ross, R.P. (1997). Non-Recombinant approaches to increase the phage resistance of starters. (Abstract) *Irish Journal of Agricultural and Food Research*. 36 258.

O'Sullivan, D., Coffey, A.G., Fitzgerald, G.F. and Ross, R.P. (1998). Sequential Non-Recombinant construction of a designer bacteriophage resistant *Lactococcus lactis* cheese starter strain. Abstract in *Proceedings of the ASM Conference on Streptococcal Genetics: Genetics of the Streptococci, Enterococci and Lactococci.* American Society for Microbiology, Washington DC, USA. 1A-12.

Coffey, A.G., Coakley, M., McGarry, A., Fitzgerald, G.F. and Ross, R.P. (1998). Increasing the phage resistance of cheese starter *Lactococcus lactis* DPC4268 in response to the emergence of a novel highly virulent phage in industry. *Actes du Colloque LACTIC-97 Lactic Acid Bacteria: Which strains? For which products?* Adria Normandie. © 1998BP2-14310 Villers Bocage, Cedex, France.

Coffey, A.G. and Ross, R.P. (1998). Cheese Starter Cultures: the bacteriophage problem. *The Irish Scientist 1998 Year Book..* No. 6, Nov 1998.

O'Sullivan, D., Coffey, A.G., Fitzgerald, G.F., Hill, C. and Ross, R.P. (1998). Design of a phage-insensitive lactococcal dairy starter via sequential transfer of naturally occurring conjugative plasmids. *Applied and Environmental Microbiology.*64 618-4622.