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Original article

Bacteriophages infecting dairy propionibacteria

M Gautier, A Rouault, P Sommer, R Briandet, D Cassin

Laboratoire de recherches de technologie laitière, INRA, 65, rue de Saint-Brieuc, 35042 Rennes cedex, France

Summary — Nineteen bacteriophages infecting *P freudenreichii* have been isolated from 32 Swiss-type cheeses. They all present a similar morphology and belong to the B1 group of Bradley's classification. According to their restriction patterns, obtained with the endonuclease *PstI*, they can be grouped into 3 clusters, 1 of which contains the majority of the phages. Sixteen of the 32 cheeses studied were infected with bacteriophages. We observed that all the cheeses made from raw milk and ripened in a warm curing room for several weeks which are known to contain high concentrations of propionibacteria, were infected with bacteriophages. Moreover, the multiplication of bacteriophages occurred in cheese during the multiplication stage of propionibacteria in a warm curing room. We have shown that raw milk contains bacteriophages and thus it can be a source of phages in factories. However, these bacteriophages are sensitive to the milk heat treatment used for cheesemaking.

bacteriophage / *Propionibacterium* / Swiss-type cheese

Résumé — Bactériophages infectant les bactéries propioniques laitières. *Dix-neuf bactériophages actifs sur* P freudenreichii *ont été isolés à partir de 32 fromages à pâte pressée cuite. Leur morphologie est similaire et permet de les classer dans le groupe B1 de la classification de Bradley. L'étude du profil de restriction de leur chromosome obtenu avec l'endonucléase* PstI *a permis de les classer en 3 groupes, dont un en rassemble la grande majorité. Seize fromages parmi les 32 étudiés étaient contaminés par des bactériophages. Nous avons remarqué que tous les fromages faits avec du lait cru, affinés en cave chaude et qui par conséquent montrent une haute concentration en bactéries propioniques, contenaient des bactériophages. De plus, la multiplication de ceux-ci se produisait parallèlement à celle des bactéries lors de l'affinage. Nous avons aussi montré que le lait cru pouvait véhiculer des bactériophages et donc constituer une voie d'entrée des phages dans les usines. Ces phages sont cependant sensibles aux traitements thermiques appliqués au lait avant la fabrication fromagère.*

bactériophage /* Propionibacterium */ fromage à pâte pressée cuite

INTRODUCTION

One of the strategies for the study of propionibacteria is the development of a cloning system for the genetic improvement of these bacteria. The development of such a system requires, on the one hand, a technique of DNA transfer and, on the other hand, a cloning vector. As no cloning vector sufficiently efficient for propionibacteria has been described, we looked for a DNA able to replicate and express itself in this bacterial genus. Plasmids are good candidates because they can replicate in the cell and can carry selection genes. However, propionibacteria harbour very few plasmids (Perez Chaia *et al*, 1988; Rehberger and Glatz, 1990). Moreover, plasmid curing experiments have failed to determine the role of these plasmids (Rehberger and Glatz, 1990). For this reason, we concentrated on the research of bacteriophages infecting the dairy propionibacteria. Bacteriophage chromosome can replicate in these bacteria and its transfer is characterized by plaques on the surface of a bacterial culture. Within the *Propionibacterium* genus, only bacteriophages infecting *Propionibacterium acnes*, a cutaneous species, have been described (Zierdt, 1974; Webster and Cummins, 1978). However, the recent finding in our laboratory of a bacteriophage infecting *P freudenreichii* indicated that these bacteria are also sensitive to bacteriophages (Gautier *et al*, 1992a). Because of the importance of dairy propionibacteria in cheese technology, it is essential to evaluate the influence of bacteriophages within this group of microorganisms.

OCCURRENCE OF BACTERIOPHAGES IN SWISS-TYPE CHEESE

We have looked for bacteriophages in Swiss-type cheese because these cheeses present a high propionibacteria population (Steffen *et al*, 1993). To this end, 32 cheese samples originating from different manufacturers were studied. To detect bacteriophages in these cheeses, 50 g of each sample were dispersed separately with an Ultraturrax blender at 20 000 rpm in 60 ml of Yel medium (Hettinga *et al*, 1968). The suspension was then centrifuged for 20 min at 3 000 *g*. After filtration on a 0.2 µm filter (Millipore), each supernatant was tested, following the Adam's method (1959), on 44 strains of *P freudenreichii* from our collection (TL: Collection du Laboratoire de Recherches de Technologie Laitière, Institut National de la Recherche Agronomique, Rennes, France). This species was used because it is the most commonly found in Swiss-type cheese. After 48 h of incubation at 30°C in jars, the presence of bacteriophages in the cheese samples was shown by the appearance of plaques on the plates. Sixteen of the 32 cheeses studied were contaminated with bacteriophages. The level of contamination ranged from 14 to 7×10^5 bacteriophages/g of cheese, depending on the cheeses and the indicator strain used for detection.

To determine whether this high frequency of bacteriophage contamination was normal, we looked for bacteriophages in different batches of the most heavily contaminated cheeses, extended over 1 to 2 months. We observed that the contamination remained constant, indicating its regularity. The production of 1 cheese (D6 cheese) was studied for more than 1 year and we observed that all the cheese samples were contaminated. Thus, it appears that bacteriophage contamination in cheeses is not an episodic but rather a chronic phenomenon.

STUDY OF BACTERIOPHAGE DIVERSITY

Knowledge of the diversity of bacteriophages is necessary to estimate their importance in Swiss-type cheese technology.

Morphology

A first approach was the study of their morphology using electron microscopy. The 19 bacteriophages studied all presented a similar morphology. As shown in figure 1, these bacteriophages have an isometric head and a noncontractile tail with a terminal plate. The head measurements of bacteriophage B22 are 48.5–50 nm from base to apex and 50–53 nm in width. The average length of the tail is *ca* 120 nm and the width *ca* 6 nm (Gautier *et al*, 1992a). Thus, they can be considered as belonging to group B1 in Bradley's classification (Bradley, 1967). It is a very common morphology since it is observed in many phages infecting different bacteria such as *Lactococcus* or *Corynebacterium* (Bauer *et al*, 1970; Accolas and Spillmann, 1979; Trautwetter *et al*, 1987). Moreover, these bacteriophages are similar to those infecting *P acnes.*

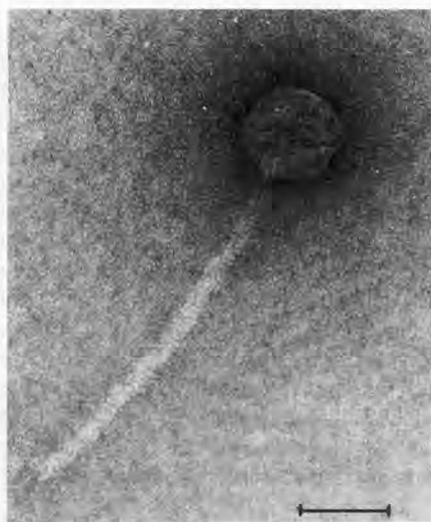

Fig 1. Electron micrograph of TL110 B7 phage (stained with 2% uranyl acetate).
Photographie en microscopie électronique du phage TL110 B7 contrasté à l'acétate d'uranyle à 2%.

Chromosome restriction pattern

Since the morphology cannot be used to differentiate these bacteriophages, we compared the restriction patterns of their DNA, cleaved with the endonuclease *PstI* (Gautier *et al*, 1995b). We observed that most of the phages had identical or related restriction patterns. Six phages presented identical restriction patterns. These similar bacteriophages were detected using the indicator strain TL110. Five phages came from different cheeses and the sixth was isolated 2 years ago in our laboratory.

According to the number of common bands, we can group these 19 bacteriophages into 3 clusters. One cluster groups together the majority of the bacteriophages (17). The 2 other groups consist of phage TL110 B3 and phage TL110 E1, respectively.

The study of these patterns shows that a cheese could be contaminated by different bacteriophages. For example, the cheese B3 contained the bacteriophages TL110B3 and TL19 E4. The cheese EI was infected by 2 different bacteriophages: phage TL11O E1 and phage TL19 E1.

Protein pattern

The analysis of the protein patterns obtained by SDS-PAGE corroborates this classification. Figure 2 shows that the pattern of bacteriophage TL110 B3 is different from the patterns obtained with the majority of the bacteriophages.

SENSITIVE STRAINS

Among 44 strains of *P freudenreichii* from our collection, only 8 were effective in phage detection and, among those, 4 were sensitive to numerous bacteriophages: TL110,

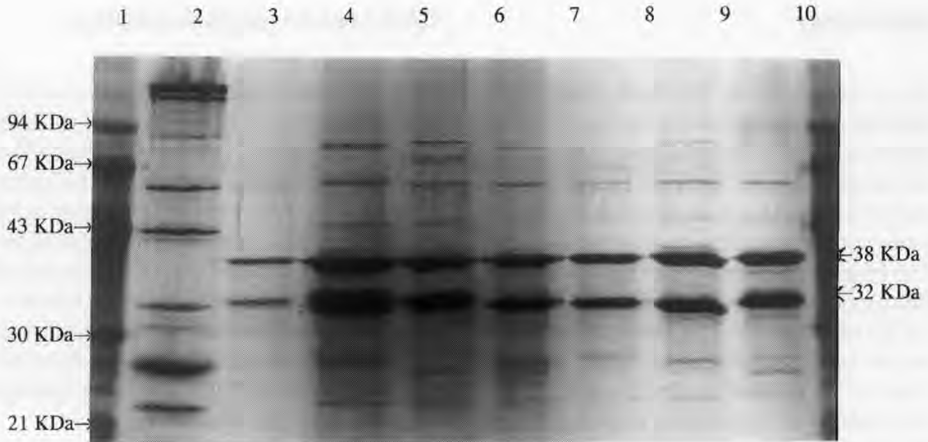

Fig 2. Patterns of structural proteins of *P freudenreichii* bacteriophages obtained by SDS-PAGE (silver nitrate staining). Lanes 1 and 10: molecular mass standards (Pharmacia); lane 2: phage TL110 E1; lane 3: phage TL303 D7; lane 4: phage TL19 E1; lane 5: phage B22; lane 6: phage TL110 D6; lane 7: phage TL110 B7; lane 8: phage TL19 E4; lane 9: phage TL19 B3.
Profils protéiques obtenus en SDS-PAGE, de plusieurs bactériophages infectant P freudenreichii. *Pistes 1 et 10 : marqueurs de masses moléculaires (Pharmacia) ; piste 2 : phage TL110 E1 ; piste 3 : phage TL303 D7 ; piste 4 : phage TL19 E1 ; piste 5 : phage B22 ; piste 6 : phage TL110 D6 ; piste 7 : phage TL110 B7 ; piste 8 : phage TL19 E4 ; piste 9 : phage TL19 B3.*

TL29, TL301 and TL302. In order to evaluate their relationship, we compared their DNA restriction pattern obtained after treatment with the infrequent cutting endonuclease *Xba*I (Gautier *et al,* 1992b). We observed that the restriction patterns of TL301 and TL302 were identical and the patterns of TL110 and TL29 were closely related. On the other hand, the other strains showed various different patterns.

Detection of bacteriophages was hampered by the lack of indicator strain. In order to avoid this problem, we tried to isolate representative strains from the cheese and to use them as indicator strains. We isolated 10 colonies from each of 4 contaminated cheeses and used them to detect bacteriophages in the corresponding filtrate. One to 3 isolates per cheese revealed bacteriophages, indicating that this method is well adapted for the detection of bacteriophages in Swiss-type cheeses.

When we studied the host range of these bacteriophages, a strong specificity with regard to the propionibacteria strains was observed. For example, bacteriophages active on the TL110 strain are active on TL29 only and these 2 strains are closely related. However, an exception must be made for phage 19, which is active on several different strains. In order to understand this close specificity, we studied the adsorption of phage B22 on 38 other *P freudenreichii* strains. We observed an adsorption of phage B22 of 3 strains only and the adsorption curves on these strains were similar to those obtained with the sensitive strains.

In order to determine if the multiplication inability of bacteriophage B22 in the 3 strains was due to a strain resistance mechanism, we looked for the restriction/modification systems (R/M) in these strains. Using a high titer bacteriophage suspension, we overcame a resistance mechanism present in 1

strain and with successive propagation of the bacteriophage on both the sensitive and the resistant strains, we demonstrated the presence of a R/M mechanism in the resistant strain (data not shown).

ORIGIN OF BACTERIOPHAGES

Bacteriophages were detected in cheeses produced (16/32) with either raw or heated milk. Figure 3 shows the number of contaminated cheeses corresponding to the milk heat treatment used. For Swiss-type cheese production, the milk can be raw or thermalized (63°C, 30 s) or pasteurized (72°C, 15 s). We observed that the proportion of contaminated cheeses was greater in cheeses made from raw milk (8/11) than in those made from milk heated at 63°C for 30 s (6/13), or at 72°C for 15 s (2/8). All the cheeses tested made from raw milk and ripened in a warm curing room for several weeks, which are known to contain high concentrations of propionibacteria, contained bacteriophages. The grated cheeses were no more highly infected than the cut cheeses. In addition, in figure 4, it can be seen that cheeses produced in large factories were less frequently contaminated than

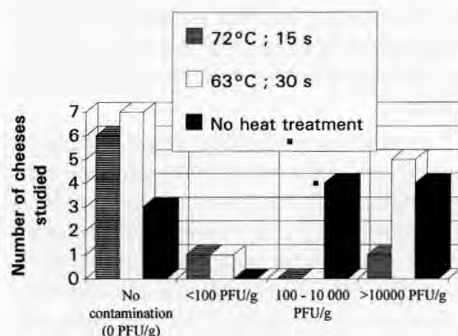

Fig 3. Number of contaminated cheeses according to the milk heat treatment.
Nombre de fromages contaminés en fonction du traitement thermique appliqué au lait.

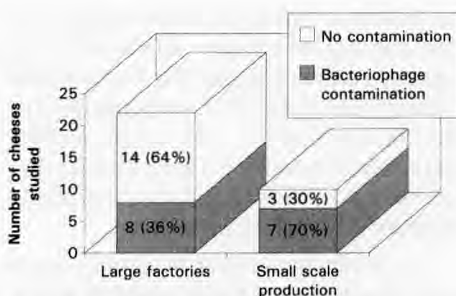

Fig 4. Percentage of contaminated cheeses according to the factory type.
Pourcentage de fromages contaminés suivant le type d'entreprise.

those resulting from small-scale production. This might, however, reflect the fact that raw milk cheeses tend to be produced in small factories.

In order to study bacteriophage multiplication during the cheese processing, we followed the production of cheese D6. This cheese is highly contaminated by bacteriophages since we found 10^3 to 10^4 PFU/g in the samples. Moreover, several batches collected over a period of 1 year were all contaminated, indicating a continual presence of bacteriophages in the factory. This cheese was contaminated with bacteriophages active against 2 strains (TL110 and TL301) which were used to detect bacteriophages during the cheesemaking. Bacteriophages were enumerated directly and, under these conditions, the detection limit was 1 PFU/ml in the liquid samples and 3 PFU/g in the solid samples. No bacteriophage was detected in the raw milk during the production process (clotting, after curd cooking, after pressing and pickling), nor after 2 weeks of ripening in the cold curing room (8°C). However, we enumerated 10^3 PFU/g of cheese after 4 weeks in the warm curing room. This observation is not surprising because bacteriophages need the multiplication of propionibacteria for their own multiplication. In order to establish the

multiplication time of the bacteriophage and the number of phages released by an infected cell, we analysed the 1 step growth of bacteriophage B22. We observed (fig 5) that the latent period is 6 h 30 long and the exponential phase is 5 h long. The number of released phages was about 60 phage particles per infected cell.

To better characterize the strains which had allowed for bacteriophage multiplication in cheese, we isolated 20 colonies of *P freudenreichii* from the D6 cheese and tested their sensitivity to the bacteriophages in the corresponding filtrate. Nine of them were sensitive. Study of the restriction patterns of their DNA revealed 4 different types, which were all different from those of the 8 indicator strains of our collection. To determine whether these sensitive strains were introduced by this starter which is widely used in France for Swiss-type cheesemaking, we isolated 20 colonies from the starter and characterized them by PFGE fingerprinting. We observed only 2 different but closely related patterns. One of these 2 strains was sensitive to the cheese D6 filtrate and had a pattern different from those of the sensitive strains isolated in the cheese. This indicates that bacteriophage multiplication can occur on either endogenous or starter-borne strains of propionibacteria. This involvement of spontaneous strains might render bacteriophage multiplication difficult to control.

To determine the primary origin of the bacteriophages, we searched for them in raw milk and in starters. We first tested 70 samples of raw milk from different regions of France. Each sample received was a blend of milk from 15 producers. As raw milk is usually poor in propionibacteria, we supposed it must be poor in bacteriophages as well. We therefore enriched the samples by inoculating them with a mixture of the 8 indicator strains. Following incubation, only 2 samples produced plaques: in the first sample, the bacteriophages were active against

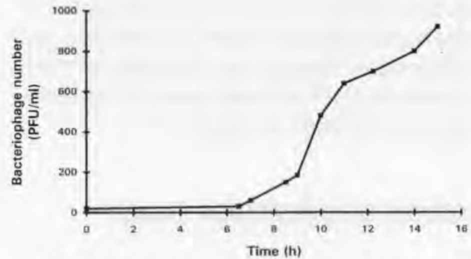

Fig 5. One-step growth curve of B22 bacteriophage.
Courbe de multiplication du bactériophage B22.

strains TL110 and TL29 and, in the second, the bacteriophages were active against strains TL301 and TL19. A similar experiment was used to detect bacteriophages in the 2 starters most frequently used in France. However, no bacteriophage active against any of the 8 indicator strains was detected.

The isolation of bacteriophages in 2 samples of raw milk indicates that milk can constitute a significant source of bacteriophages. In contrast, the fact that no bacteriophage could be detected in the 2 samples of propionibacteria starter examined favours the view that commercial starters are not a major source of bacteriophages. This, however, does not exclude the possibility that propionibacteria harbour prophages and a study of the lysogenic status of these bacteria is of the utmost importance.

In order to understand why the cheeses made with pasteurized milk were less contaminated than the other cheeses, we tried to determine if the milk heat treatments have an effect on bacteriophage inactivation. We then studied the heat sensitivity of bacteriophage B22. These experiments were carried out in milk. As shown in figure 6, this bacteriophage is sensitive to thermalization and even more so to pasteurization.

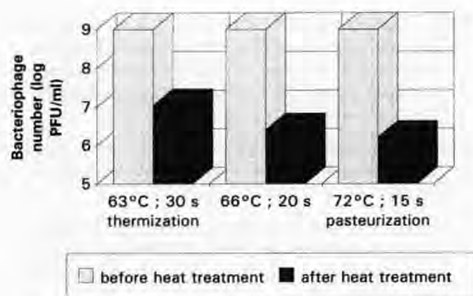

Fig 6. Heat sensitivity of B22 bacteriophage.
Sensibilité à la chaleur du bactériophage B22.

CONCLUSION

It may be questioned whether it is useful to work on propionibacteria bacteriophages. First, it appears important to determine if they have an impact on the propionic flora in Swiss-type cheese. Although propionibacteria bacteriophages are frequent in these cheeses, their impact on cheese technology and quality is probably limited. We have shown that bacteriophages coexist in the cheese with an abundant population of phage-sensitive cells, indicating that propionibacteria destruction is only partial. Because of the solid structure of Swiss-type cheese, bacteriophages cannot propagate throughout the cheese. Consequently, their multiplication occurs in different sites and only partially hampers the propionibacteria development. The fact that only a relatively low population of bacteriophages has been found in cheese samples suggests that they probably do not significantly disrupt the cheesemaking process. However, it is possible that the number of bacteriophages in the cheese has been underestimated. Indeed, we have recently used a new method to extract bacteriophages from the cheese: the cheese is pressed and the bacteriophages are found in the liquid obtained. Compared with the results obtained with the Ultraturrax blender, the number of bacteriophages detected is 10 times higher with the press. Consequently, it is possible to find up to 10^7 PFU/g of cheese. It may be asked whether bacteriophages present in the cheese have a role in bacteria autolysis, and therefore a positive effect on the ripening.

Secondly, the study of propionibacteria bacteriophages is interesting since they can be useful tools for the development of engineering techniques for propionibacteria. For example, the use of phage DNA as a replicon efficient in propionibacteria allowed us to optimize the electrotransformation of these bacteria. We obtained a transformation efficiency of about 7×10^5 transformants/μg of DNA (Gautier *et al*, 1995a) and this protocol can be used to develop an efficient genomic cloning system with propionibacteria. As seen previously, bacteriophages can be used for the detection of restriction/modification systems.

The development of genetically modified microorganisms used in the food industry tends towards the integration of new genes on the chromosome. One strategy is the use of a vector carrying the insertion site of a temperate bacteriophage. The construction of such a vector requires the isolation of a temperate phage infecting the dairy propionibacteria. Preliminary experiments showed that some strains harboured bacteriophage DNA on their chromosome.

Finally, bacteriophage genes can be used for the improvement of technological properties. It is conceivable to use a phage lysin gene to control and improve bacteria autolysis in cheese. To this end we are attempting to clone the lysin gene of phage B22 in *E coli*.

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