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Original article

Bacteriophages of dairy propionibacteria

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Abstract — Two types of bacteriophage (phage) infecting dairy propionibacteria (PAB) were isolated from Swiss-type cheeses. One belongs to Bradley's classification group B1 and the other is, to our knowledge, the first infective filamentous virus described in Gram positive bacteria. Both types of phages were present in several different Swiss-type cheeses. A possible source has been shown to be raw milk where they are found in small quantities. The B1 type phage can be harboured by PAB in the prophage state. DNA hybridization experiments showed that all PAB B1 phages are closely related, although they were isolated from different cheeses and probably have a common origin. As sensitive strains can constitute the predominant PAB flora at the end of cheese ripening, it seems obvious that phage did not disturb the development of this flora and consequently did not have an unfavourable effect on the organoleptic properties of Swiss-type cheeses. This information concerning phages allowed us to develop an efficient cloning system for *Propionibacterium freudenreichii*. © Inra/Elsevier, Paris.

bacteriophage / *Propionibacterium freudenreichii*

Résumé — Des bactériophages de bactéries propioniques laitières. Deux types de bactériophages (phages) infectant des bactéries propioniques laitières (BPL) ont été isolés à partir de fromages à pâte pressée cuite. L'un appartient au groupe B1 de la classification décrite par Bradley alors que l'autre est à notre connaissance le premier virus filamenteux infectieux décrit à ce jour chez une bactérie à Gram positif. Les deux types de phages ont été identifiés dans plusieurs fromages à pâte pressée cuite différents. Leur origine pourrait être le lait cru où de faibles quantités de phage ont été détectées. Les phages du groupe B1 peuvent être portés par les BPL à l'état de prophage. L'hybridation de l'ADN montre que tous les phages de BPL du groupe B1 sont étroitement liés, bien qu'ils aient été isolés de fromages différents. Ils ont donc probablement une origine commune. La flore propionique dominante à la fin de l'affinage des fromages peut être constituée d'une souche de BPL sen-

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sible. Il semble dès lors évident que les phages n'en perturbent pas le développement et par conséquent n'ont pas d'effet néfaste sur les propriétés organoleptiques des fromages à pâte pressée cuite. Ces données nous ont par ailleurs permis de mettre au point un vecteur de clonage efficace chez *Propionibacterium freudenreichii*.

bactériophage / *Propionibacterium freudenreichii*

1. INTRODUCTION

Phages infecting dairy PAB were first described by our group in 1992 [9]. Prior to this date, only phages infecting *P. acnes* had been detected. These phages were similar to numerous other phages described in the literature since they belong to group B1 of Bradley's classification [5]. Subsequent studies were performed with the aim of typing the strains of *P. acnes* which are involved in cutaneous infections [28]. Problems in detecting phages for dairy PAB are probably related to the difficulty of selecting sensitive strains. Phages infecting other bacteria involved in cheese making, particularly starter bacteria such as *Lc. lactis*, were studied much earlier because they cause significant disruption in the fermentation. The progress of propionic fermentation, which is a secondary fermentation in cheese ripening, was probably of less concern for dairy technologists and any incorrect development of the PAB flora during ripening was rarely linked to the presence of phage. Efforts in this laboratory to improve a cloning system for dairy PAB has led to the search for phage infecting these bacteria with the objective of using the phage DNA as a vector. DNA transfer is easily detectable by plaque formation. Moreover, a temperate phage could be an attractive tool for cloning experiments.

We have searched for phages in Swiss-type cheeses, which are the major ecological niche of dairy PAB. This report is a review of the present knowledge on the phages infecting dairy PAB.

2. MATERIALS AND METHODS

2.1. General procedures

Bacterial strains and culture conditions, phage detection, electron microscopy, extraction and digestion of phage DNA, bacterial genomic DNA preparation, digestion and pulsed-field gel electrophoresis (PFGE) have been previously published [11, 12].

2.2. Optimum conditions for B22 phage adsorption

The method described by Trautwetter et al. [26] was employed.

2.3. Kinetics of phage destruction

Several flasks, each containing 100 mL of pasteurized milk, were inoculated with the phage suspension (10^8 pfu·mL^{-1}) and incubated following a heating cycle reproducing the successive temperatures used in Swiss-type cheese fabrication up to the brine salting stage. At different time points, one flask was taken for phage enumeration. A heat exchanger (Vulcain) with a Micropore III P (Coreci, Lyon, France) automatic control unit was used.

2.4. Induction of lysogenic PAB with UV-irradiation or mitomycin C

The method described by Chopin et al. [8] was used.

2.5. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The method described by Schägger and von Jagow [22] was followed.

2.6. Dot blot and Southern blot experiments

The methods described by Anderson and Young [1] for the dot blot and by Southern [24] for the Southern blot were employed.

2.7. Phage DNA probe labelling and hybridization

The multiprime DNA labelling system (Amersham, Buckingham, UK) was used following the supplier's instructions.

3. RESULTS

3.1. Phage characteristics

Two different types of phages infecting PAB were isolated from Swiss-type cheeses. The first type belongs to group B1 of Bradley's classification, or to the siphoviridae of the International Committee on Taxonomy of Viruses [18]. These phages have an isometric head and a noncontractile tail with a terminal plate and have already been described in more detail in a previous paper [9]. This type of phage is very common and can be found in soft cheeses since they infect lactic acid bacteria such as *Lc. lactis* [2] and *Corynebacterium* [26], which is a genus closely related to *Propionibacterium*. Their genome consists of a linear double-stranded cohesive ended DNA molecule, 35–40 kb in size.

The other type of phages infecting dairy PAB is significantly rarer and it has not, as yet, been studied in detail. As shown in *figure 1*, it is a filamentous phage 620 nm long and 12 nm wide. To our knowledge, it is the first filamentous infective phage identified in Gram positive bacteria. A filamentous phage infecting *Clostridium acetobutylicum* reported previously by Kim and Blaschek [15] was considered to be a defective phage. Like other phages infecting PAB, the phage shown in *figure 1* was isolated from various Swiss-type cheeses from one

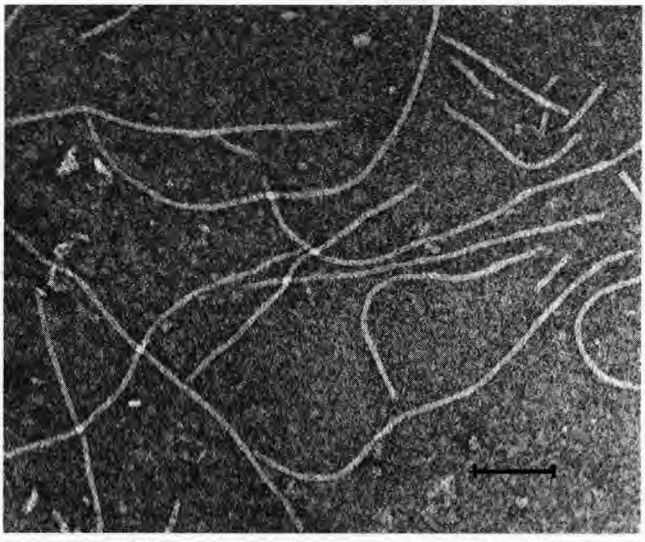

Figure 1. Electron micrograph of a filamentous phage stained with 2 % uranyl acetate. The bar indicates 100 μm.

Figure 1. Micrographie électronique d'un phage filamenteux contrasté à l'aide d'acétate d'uranyl 2 %. La barre indique 100 μm.

factory that we have been studying for several years. Its genome consists of a single strand of nucleic acid, 3.4–4 kb in size and nucleotide sequencing is in progress. Analysis of the protein patterns obtained by SDS-PAGE shows one small major protein of 6 kDa (*figure 2*). Such a major protein has previously been described in other filamentous phages [15, 19]. At present very little information on this filamentous phage is available and more research into its physiology and ecology is required.

3.2. Diversity and host spectrum

So far, the phages studied have shown similar morphology and protein patterns; their diversity has been estimated from DNA restriction pattern only. With regard to group B1 phages, the *Pst*I DNA restriction pattern of 13 phages isolated from cheeses showed considerable similarity with each other [11]. Identifiable differences in banding patterns allowed these phage to be roughly arranged into 3 groups. One was well represented, since it contained the majority of phages (9 phages), and the two other groups were composed of 2 phages each. In order to determine if the phages were closely related, hybridization experiments were conducted. One phage representative of each group was chosen and its DNA hybridized with that of the other phage. Hybridization patterns, shown in *figure 3*, confirm the close relationships between these phages. This high homology is not surprising because, during their multiplication cycle, different phages can exchange modules (interchangeable genetic elements) [3]. Moreover, all these phages have been isolated from cheeses, using the

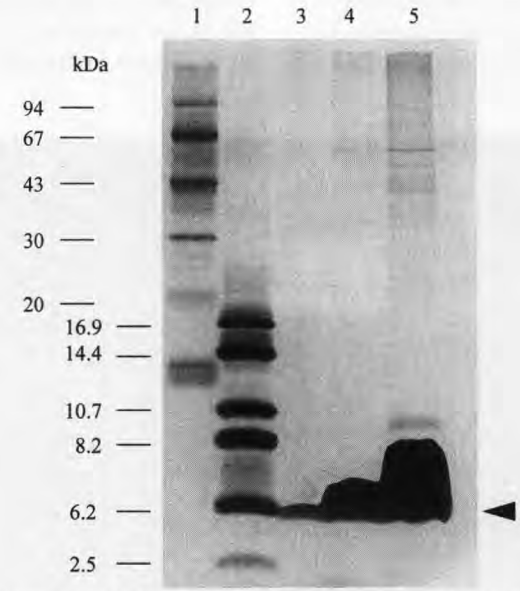

Figure 2. SDS-PAGE analysis of phage B5 proteins using silver staining. 1 and 2: molecular mass markers, 3 to 5: phage B5 SDS extract. The protein quantity in each lane was 3: 2 µg, 4: 20 µg and 5: 100 µg. The 6-kDa major coat protein is indicated by the arrow.

Figure 2. Analyse des protéines du phage B5 par EGPA-SDS et coloration argentique. 1 et 2 : marqueurs de masse moléculaire, 3 à 5 : extrait SDS du phage B5. La quantité de protéines déposée par piste est : 3 : 2 µg, 4 : 20 µg et 5 : 100 µg. La flèche indique la protéine majoritaire d'enveloppe de 6 kDa.

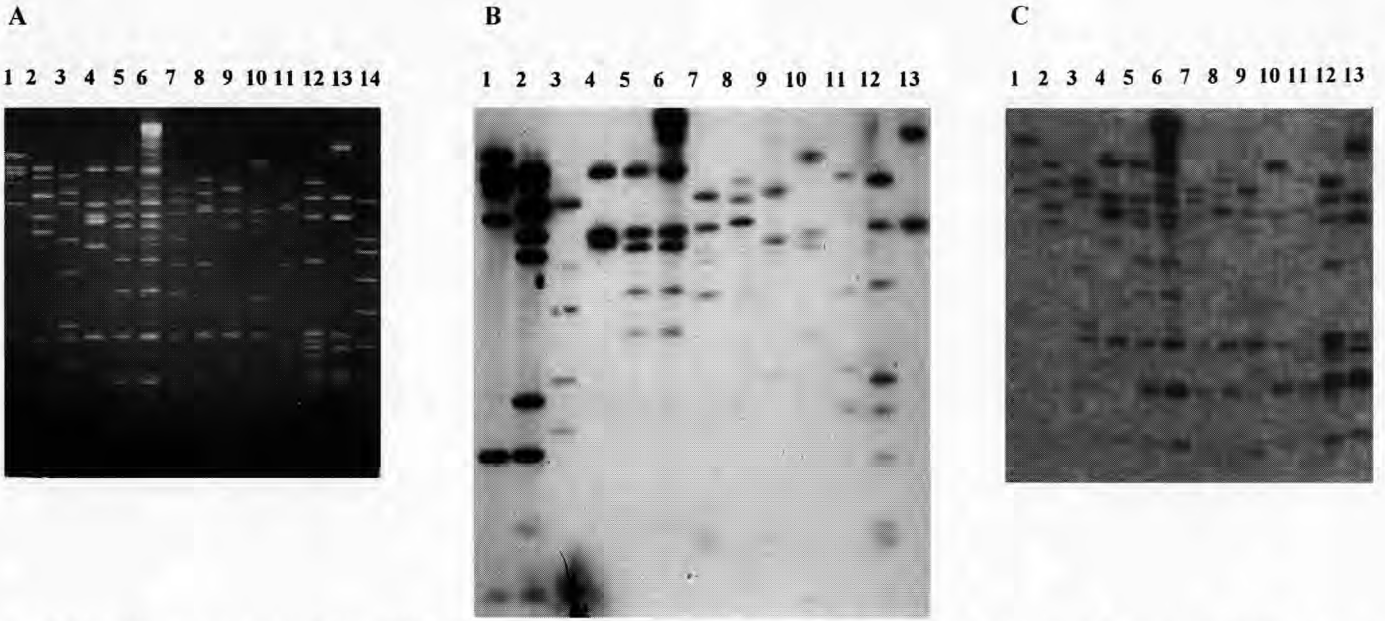

Figure 3. Hybridization analysis of phage DNA. The group number to which the phage belongs is given in brackets. **A.** Agarose gel electrophoresis of *PstI* digested DNAs of the 13 phages. **B** and **C.** Hybridization analysis of the same DNAs using (B) the DNA of phage TL110D6 (2) or (C) the DNA of phage TL110E1 (3) as a probe.
1: ϕ TL110B3 (3); 2: ϕ TL110E1 (3); 3: ϕ TL110B5 (1); 4: ϕ B22 (1); 5: ϕ TL110B7 (1); 6: ϕ TL110M (1); 7: ϕ TL110E6 (1); 8: ϕ TL19B3 (1); 9: ϕ TL19E4 (1); 10: ϕ TL18X (1); 11: ϕ TL303D7(1); 12: ϕ TL110D6 (2); 13: ϕ TL19E1 (2). Lane 14 in A: molecular weight marker Raoul (Appligène).

Figure 3. Analyse d'ADN phagique par hybridation. Le groupe auquel le phage appartient est indiqué entre parenthèses. **A.** Électrophorèse sur gel d'agarose de l'ADN des 13 bactériophages digéré à l'aide de *PstI*. **B** et **C.** Analyse par hybridation des mêmes ADNs à l'aide (**B**) de l'ADN du phage TL110D6 (2) ou (**C**) de l'ADN du phage TL110E1 (3) comme sonde.
1 : ϕ TL110B3 (3) ; 2 : ϕ TL110E1 (3) ; 3 : ϕ TL110B5 (1) ; 4 : ϕ B22 (1) ; 5 : ϕ TL110B7 (1) ; 6 : ϕ TL110M (1) ; 7 : ϕ TL110E6 (1) ; 8 : ϕ TL19B3 (1) ; 9 : ϕ TL19E4 (1) ; 10 : ϕ TL18X (1) ; 11 : ϕ TL303D7(1) ; 12 : ϕ TL110D6 (2) ; 13 : ϕ TL19E1 (2). Piste 14 (**A**) : marqueurs de taille Raoul (Appligène).

small number of sensitive strains we have in our strain collection. This limited number of sensitive strains is probably an obstacle in detecting different phages.

All these phages seem to be temperate and are probably derived from a common ancestor. It is highly probable that the phage diversity in the environment is as large as Bruttin et al. [7] have shown for phages infecting *S. thermophilus*. Moreover, in previous work using PFGE restriction analysis patterns, it has been observed that the genetic diversity of dairy PAB in raw milk is extremely wide (unpublished data). Indeed each isolate showed a specific pattern. It is conceivable that this diversity may also be observed with phages infecting this bacterial group.

Concerning the filamentous phage, their genome is made up of a single-stranded nucleic acid, so we cannot obtain a restriction pattern with the endonucleases classically used. Consequently, from the nucleic sequence of one of them, we intend to choose 2 primers framing a small sequence in order to amplify the same fragment in all filamentous phages. The comparison of the different sequences should give us some information on their diversity.

3.3. Host spectrum

All phages studied showed a very narrow host spectrum. They are able to infect only one or two strains of PAB and when two host strains are sensitive to the same phages, this indicates a close relationship between strains. However, it is possible to find new sensitive hosts from the PAB found in cheese from which the phage themselves were isolated [11].

This narrow host spectrum may be due to the phages' inability to adsorb on the cell wall. In order to verify this hypothesis, the adsorption capacity of phage B22 on 37 strains of PAB was studied. This phage could adsorb on only 5 strains; two were able to propagate the phage and were closely related. The 3 other strains were not sensitive to this phage. Moreover, 2 other strains of PAB known to be sensitive to several phages closely related to the B22 phage were unable to adsorb phage B22. Unlike phages infecting lactic acid bacteria [16], the adsorption of the B22 phage was not dependent on the presence of 10 $mmol \cdot L^{-1}$ or 100 $mmol \cdot L^{-1}$ mono- or divalent cations ($CaCl_2$, $MgSO_4$, $ZnCl_2$, KCl, $NiCl_2$, LiCl, $FeCl_2$, $CuCl_2$, $CuSO_4$, $MnSO_4$, K_2SO_4, $FeSO_4$, $ZnSO_4$, $Co(NO_3)_2$).

Even if the phages are not easily adsorbed onto PAB cell wall, this observation is not sufficient to explain their very narrow host spectrum. The lysogenic status of PAB (see below) explains part of their phage resistance because they are resistant to superinfection with a phage closely related to the integrated prophage. It is necessary to determine the prevalence of lysogeny and if lysogenic strains harbour different prophages on their chromosome.

When the host spectrum of our phages was established, a difference in the efficiency of plating was observed when some phages were tested on 2 different strains. Using cross propagations with high titer phage suspensions, the presence of a R/M system in 3 strains has been demonstrated (the demonstration for two of them is shown in *table I*). However, from these experiments, it is difficult to draw any conclusion about the distribution of R/M mechanisms among dairy PAB, as the phages were active on 10 strains only. For this reason, we have used biochemical tests, in collaboration with A. Janulatis (Institute of Biotechnology of Vilnius, Lithuania) and have screened 50 strains for the type II R/M system. Only one strain showed *Pst*I activity while another showed *Nae*I activity. However, it is possible that PAB may have a type I or type III restriction system as recently described in *Lc. lactis* [23]. They may also have other phage resistance mechanisms, such as the abortive infection systems which have been described in *Lc. lactis* [20].

Table I. Demonstration of the presence of the R/M mechanisms in *P. freudenreichii* strains TL176 and TL18.
Tableau I. Mise en évidence des mécanismes de R/M chez les souches TL176 et TL18 de *P. freudenreichii*.

Indicator strain	Efficiency of plating[a] of phage ϕDi propagated on strain[b]		
	TL176	TL176 TL18	TL176 TL18 TL176
TL176	1	3×10^{-8}	1
TL18	4×10^{-9}	1	3.3×10^{-9}

[a] Pfu·mL^{-1} formed on the indicator strain divided by pfu.mL^{-1} formed on the propagating strain / Pfu·mL^{-1} obtenus avec la souche indicatrice divisé par pfu.mL^{-1} obtenus sur la souche de propagation.
[b] When more than one strain is indicated, the phage was propagated successively as indicated by the strain order / quand plus d'une souche est indiquée, le phage était successivement propagé dans l'ordre où sont données les souches.

3.4. Origin of phage in dairy technology

3.4.1. Raw milk

Like lactococcal phages in the cheese industry, raw milk can constitute a source of PAB phages in the Swiss-type cheese industry [11]. This observation is not surprising since raw milk can contain a high level of PAB [25]. In order to evaluate the colonization of Swiss-type cheese factories by PAB phages, the distribution of a phage which was regularly found to be present in the cheese of one particular factory has been studied. A sensitive strain was used to detect this phage directly from the samples (the detection limit was 1 pfu·mL^{-1} in liquid samples and 3 pfu·mL^{-1} in solid samples). The samples were also incubated with the sensitive strain in YEL medium [14] for 4 d to increase the sensitivity of phage detection (in this case, the detection limit was 0.1 pfu·g^{-1} of sample). Phages were detected only in raw milk (in one experiment) and in cheeses; no phage was detected in curd or in cooked curd after pressing and salting. Even if phages enter the factory via raw milk, their propagation can be limited by their destruction during cooking of the curd.

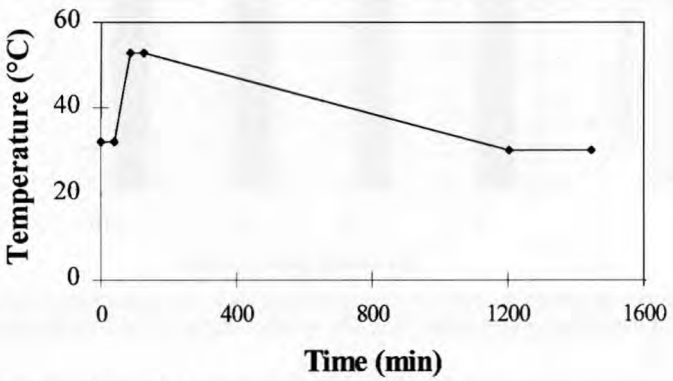

Figure 4a. Heating cycle used in Swiss-type cheese manufacture.
Figure 4a. Cycle thermique utilisé dans la fabrication des fromages à pâte pressée cuite.

Recently, the kinetic destruction (*figure 4a*) of these phages during cheese making has been studied. Two types of phages infecting PAB and isolated in the cheese factory were studied. The kinetic destruction graph of the filamentous phage, B5, (*figure 4b*) showed that it could not withstand the heat treatment as the phage number decreased by 4 log cycles in 413 min. The B1 group phage was much more resistant to heat treatment since of < 1 log was observed after 24 h (1 440 min) of heating. However, the studies showed that the level of phages in raw milk used in the factory for the manufacture of Swiss-type cheeses is very low (< 1 pfu·mL^{-1}): for its detection, it was necessary to add sensitive strains to raw milk for the phage to multiply. It is likely that the number of phages remaining in the cooked curd is below 0.1 pfu·mL^{-1}, and probably even lower after elimination of the whey. Unlike lactococcal phages, which are propagated in the factory during whey drainage, PAB phages, which are probably destroyed during the heating process, cannot spread within the factory.

Consequently, it is highly probable that the level of PAB phages in cheese at the beginning of ripening is relatively low since no phage can be detected in the cheese body before ripening. The detection limit is 0.1 pfu·g^{-1} of cheese, although Swiss-type cheeses can contain up to 7×10^5 cfu·g^{-1} PAB [11]. The appearance of phages occurs during ripening in the temperate curing room (15–16 °C) and continues throughout the ripening period in the warm room (22–24 °C). In so far as the phages are probably unable to propagate within the cheese body, it is surprising that, with such a low level of phages in the cheeses just before ripening, it is possible to observe high levels (10^5 pfu·g^{-1} cheese) after ripening. It is suspected that PAB liberate prophages during the retain growth during ripening. This hypothesis was supported by the fact that cheese from the factory we have been studying for 6 years is regularly infected by the same phage. Moreover, different phages from various Swiss-type cheeses made in France showed a closely related restriction pattern, indicating a common ancestor. The only common point between these cheeses is the PAB starter used for their manufacture. In France, the major starter market is held by only one starter producer and only a few strains of PAB are used in the production of most Swiss-type cheese.

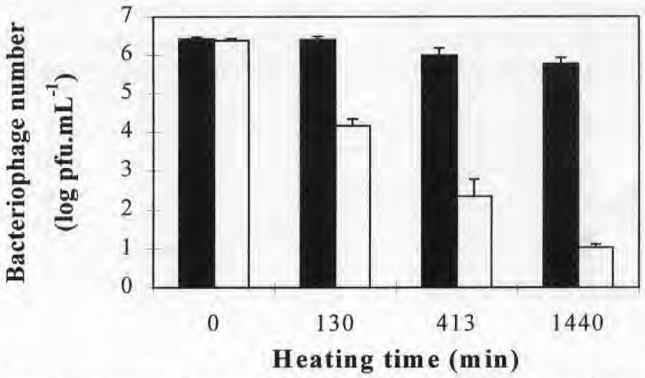

Figure 4b. Kinetics of phage destruction during heating in Swiss-type cheese manufacture. (■) Phage B22, (□) Filamentous phage (phage B5). The standard deviations of 3 independent experiments are shown.

Figure 4b. Cinétique de destruction des phages par le traitement thermique lors de la fabrication des fromages à pâte pressée cuite. (■) Phage B22, (□) phage filamenteux (phage B5). L'ecart type de 3 expérimentations indépendantes est indiqué.

3.4.2. Lysogeny

In order to determine whether PAB are capable of introducing phages by way of lysogeny, we have studied the lysogenic status of PAB. Since the conditions of prophage induction are species and strain dependent and it is difficult to detect the induced prophages since indicator strains are difficult to obtain, another method for detecting the lysogenic strains was adopted. The strains with a genome which showed homology with the DNA of some phages studied in our laboratory were selected. This idea was based on the fact that Brüssow and Bruttin [6] reported that the DNA of temperate phages of *St. thermophilus* showed homology with the DNA of virulent phage.

Eight typical phages isolated from Swiss-type cheeses belonging to group B1 of Bradley's classification, were chosen and their genomes were used as probes. The phage DNAs were hybridized by dot blot to the genomic DNA of 90 PAB strains, chosen according to their origin (starter-borne strains, strains isolated from milk or cheeses, strains obtained from international collection, etc.). Thirty three per cent of strains showed homology with phage DNA.

In order to eliminate the possibility that the strains chosen were contaminated by virulent phages, the location of the putative prophage on the bacterial chromosome was also investigated. The bacterial DNAs, showing homology with one or more phage DNAs, were digested with *Xba*I endonuclease which cuts the chromosome into several fragments [13]. The patterns obtained on PFGE showed 10–16 bands ranging in size from ~10 to 1 000 kb.

Hybridization experiments, with phage DNA as the probe, were performed by Southern blot analysis (*figure 5*). This confirmed the results obtained with the dot blot hybridization. Only 4 of the 30 bacterial DNAs giving a positive signal in the dot blot hybridization did not hybridize with the corresponding phage DNA probe.

The four strains of PAB showing the strongest homology were selected. These strains were then treated with mutagenic agents (UV or mitomycin C). The resulting

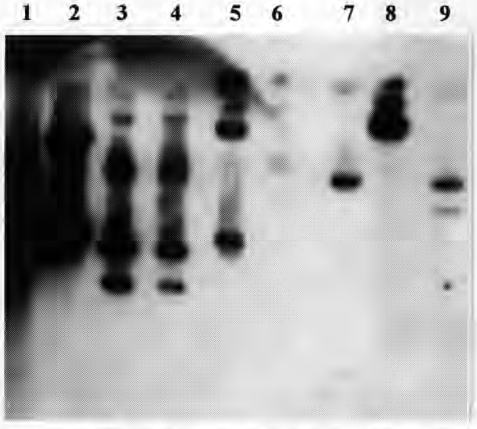

Figure 5. Hybridization analysis of the *Xba*I restriction patterns of propionibacteria strains which had given positive results in the dot blot experiments. The probe used was the DNA of phage TL110B3. 1: phage TL110B3; 2: TL146; 3: TL22; 4: TL24; 5: TL41; 6: TL100; 7: TL101; 8: TL105; 9: TL106.

Figure 5. Analyse par hybridation du profil de restriction *Xba*I des souches de propionibactéries donnant des résultats positifs lors des expériences de dot-blot. La sonde utilisée est l'ADN du phage TL110B3. 1 : phage TL110B3 ; 2 : TL146 ; 3 : TL22 ; 4 : TL24 ; 5 : TL41 ; 6 : TL100 ; 7 : TL101 ; 8 : TL105 ; 9 : TL106.

filtrates were tested on 10 strains sensitive to phages. Plaques were only obtained with one sensitive strain and consequently, the temperate phages obtained could be easily studied. In order to determine if the other strains showing homology with the phage DNA were really lysogenic, 3 of those which gave a significant hybridization signal were studied more closely. After induction, electron microscopy showed entire and defective phage particles, thus proving the lysogenic status of these PAB strains. The restriction patterns of the *Pst*I–digested temperate phages were compared and it was observed that one of these temperate phages was closely related to the *P. freudenreichii* phage B22 previously isolated from a Swiss-type cheese (*figure 6*) [9]. Phage B22, and other related phages isolated from cheeses, gave rise to clear plaques and consequently seemed to be virulent. Their similarity with a temperate phage casts some doubt on their virulent nature. In order to determine their real nature, bacteria from the centre of a plaque produced by phage B22 were collected and their lysogenic status determined. Induction of the cultures with UV-radiation gave rise to the liberation of a temperate phage identical to the B22 phage.

It appears that the majority of PAB phage, isolated from Swiss-type cheese in this laboratory over several years, are temperate and probably originate from lysogenic PAB. In order to understand why these phages are closely related, whether these lysogenic bacteria come from the starter culture or from the natural raw milk flora should be determined.

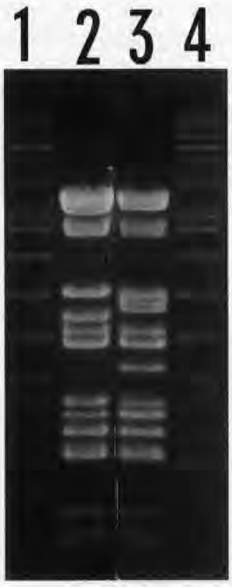

Figure 6. Agarose gel electrophoresis of *Pst*I–digested DNAs of ϕB22 phage and ϕTL146P induced phage. 1 and 4: DNA molecular weight marker Raoul (Appligène); 2: ϕTL146P; 3: ϕB22.

Figure 6. Électrophorèse sur gel d'agarose de l'ADN du phage ϕB22 et du phage induit ϕTL146P après digestion par *Pst*I. 1 et 4 : marqueurs de taille Raoul (Appligène) ; 2 : ϕTL146P ; 3 : ϕB22.

4. CONCLUSION

Phages infecting dairy PAB are of interest because they have only recently been discovered and little is known about them. It is necessary to obtain fundamental knowledge about their physiology, genetics and ecology and to determine their role in cheese technology.

The detection of filamentous phages infecting PAB is very interesting in so far as most of the information about this type of phage is from Gram negative bacteria, in particular, *Escherichia coli*, *Xanthomonas oryzae*, *Salmonella typhimurium*, *Pseudomonas aeruginosa* and *Vibrio parahaemolyticus* (for a review, see [19]). Until now only one filamentous phage infecting a Gram positive bacterium, *Clostridium acetobutylicum* has been described [15].

The interest in such a study is not the development of a method of strain typing as in the past for *P. acnes* [27], since more efficient molecular techniques are now available for this purpose [13]. It is possible to consider the use of phages as lytic agents of dairy PAB during the ripening of Swiss-

type cheeses. These viruses are found in the cheese body and they need the growth of PAB for their multiplication. However, the lysis of these bacteria occurs only during ripening in the warm room. Consequently, it would seem feasible to induce different degrees of lysis by varying the bacteria/phage ratio. In this context, future experiments will consist of producing, on a pilot scale, Swiss-type cheeses with or without added phages.

The use of PAB as starters is a fast-growing industry. In France, the inoculation of milk for the production of Swiss-type cheeses is on the increase [21]. A significant portion of the world production of vitamin B_{12} results from the use of *P. freudenreichii* in fermentation. The growing consumer demand for natural food ingredients and the more efficient performance of new fermentation processes have revived research and industrial interest in the biological production of propionic acid [4]. Finally, many researchers now consider PAB as potential probiotics [17]. Their production on a large scale requires careful control of the fermentation process and, consequently, knowledge of the phages infecting these bacteria.

In this laboratory, the detection of phages infecting *P. freudenreichii* contributed to the development of an efficient DNA transfer technique in this species. The use of phage DNA as an effective replicon in PAB allowed optimization of electrotransformation of these bacteria leading to a transformation efficiency of about 7×10^5 transformants μg^{-1} of DNA [10]. Now, using the DNA of phage which are active on other strains, it is possible to transform several strains of PAB, some of which are used in biotechnology. A cryptic theta-replicating plasmid of *Propionibacterium freudenreichii* was successfully used to construct a cloning shuttle vector, pB4 (12.5 kb), which was partly based on a *E. coli* plasmid carrying the chloramphenicol resistance determinant. It is expected that this will facilitate the study of the *Propionibacterium* genome. In order to allow the expression of antibiotic resistance genes in *P. freudenreichii*, we have added upstream from this gene a promotor originating from the same species. The localization of the sites attp and attb allows the insertion of a prophage on the bacterial chromosome and this should permit the construction of an integrative-type vector which will, in turn, facilitate the insertion of foreign genes into the bacterial chromosome.

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