The Characteristics, Detection and Control of Bacteriophage in Fermented Dairy Products

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발효유제품에서 박테리오파지의 특성, 검출과 제어

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Abstract

This study was to review the classification, detection and control of bacteriophage in fermented dairy products. Bacteriophage has lytic and/or lysogenic life cycles. Epidemiologically speaking, detected major phages are c2, 936 and p335. Among them p335 has been the largest concern in dairy industry. Traditionally, various analytical technologies, such as spot, starter activity, indicator test, ATP measurement and conductimetric analysis, have been used for the phage detection. In recent years, advanced methods such as flow cytometric method, petrifilm, enzyme linked immunosorbent assay (ELISA) and multiflex PCR diagnostic kit have been developed. The phage contamination has been controlled by using heat, high-pressure treatment, and the combinations of heat and pressure, and/or chemical. Also some starter cultures with phage-resistant character have been developed to minimize the concentration of phages in dairy product. Bacteriophage inhibition media such as calcium medium was also mentioned. To prevent the contamination of bacteriophage in dairy industry, further researches on the detection and control of phage, and phage resistant starters are necessary in the future.

Key words : bacteriophage, classification, detection, control, dairy products

Introduction

Fermented dairy foods offer unique opportunities and challenges in nutrition and health that are unavailable in other foods (Sellars, 1981). Consumers now realize the importance of these foods for maintaining high nutrition and health (Sellars, 1981). The success of the fermentation process depends primarily on the acid production of the starter culture. It is of course difficult to make good cheese unless the starters give consistently uniform acid production, and when the required acidity is achieved it can be considered the question of flavor at maturity (Lawrence *et al.*, 1978).

Lactic acid bacteria (LAB), such as lactococci, lactic streptococci and lactobacilli, are commonly used on an

industrial scale for the production of fermented foods, such as cheese and yogurt (McGrath *et al.*, 2004a). The susceptibility of these bacteria to infection by bacteriophages that are omnipresent in the dairy environment can have serious economic consequences for the dairy industry (Binetti *et al.*, 2005).

Bacteriophages cause lysis of the starter bacteria and can lead to slow or failed milk acidification and the concomitant economic loss in the dairy industry. The problem is the difficulty of bacteriophages eradication from dairy plant. They resist during pasteurization and have short latent period. Once they emerge in a vat, they propagate throughout the dairy plant (Szczepanska *et al.*, 2007). The exact origin of bacteriophages is not yet known (Madera *et al.*, 2004). Only raw milk and the lysogenic starter culture are the clearly identified source (Szczepanska *et al.*, 2007; Josephsen and Neve, 2004; Bruttin *et al.*, 1997; Brüssow *et al.*, 1994). In the dairy industry, the phage ecology is dominated by three phage

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species (McGrath *et al.*, 2004a). The early detection of bacteriophages is of great importance as lactococcal phage population level of 10^5 to 10^7 PFU/mL in starter media or milk is an indication of a potential problem (Svenson and Christiansson, 1991).

In this review, different techniques of detection and the future requirement to fulfill the dairy industry requirements are discussed. The different treatments to control bacteriophages are also mentioned. The review is covering most of the required information to give the reader a whole picture of the bacteriophage classification, origin, life cycle, epidemiology and research future trend for the early detection as well as the bacteriophage control in the dairy plant.

Classification

Different classifications have been proposed to offer reliable criteria for bacteriophage classification (Sanlibaba and Akçelik, 2005). Traditionally bacteriophages are classified by morphology, host range and serology. Recently, DNA and protein information of the bacteriophages is used for the classification.

Morphology

It is a classical method for bacteriophage classification relying on physical shape basis. By direct microscopic examination, bacteriophages are classified on the basis of head size and shape (Jarvis, 1989). All known phages of lactic acid bacteria belong to the order caudovirales (tailed phages) (Brüssow and Hendrix, 2002), where they are further subdivided on the basis of tail morphology into 3 morphotypes, Myoviridae (A) which exhibit contractile tails, Siphoviridae (B) which have non-contractile tails and Podoviridae (C) which have short non contractile tails (McGrath *et al.*, 2004a). These three families are further subdivided on head size and shape into isometric, small prolate and large prolate (elongated) heads to morphotypes 1, 2 and 3, respectively (Josephsen and Neve, 2004). The majority of phages classified into date that infect lactococcal, *Lactobacillus* species belong to the Siphoviridae B1 or B2 morphotypes. However, the bacteriophages attacking *Streptococcus thermophilus* are only reported to have the B1 Siphoviridae morphotype (McGrath *et al.*, 2004a).

Host range

The phage infection process requires a phage receptor on the bacterial surface and a receptor binding protein

(RBP) located on the distal part of phage tail. The infection starts after a specific binding between RBP and the phage receptor (Duplessis *et al.*, 2006). This determines the host range of the bacteriophages and modification to any of those structure may affect phage host interaction (Duplessis *et al.*, 2006). Variability of the host range among different bacteriophages is well reported (Daly, 1983; Heap and Jarvis, 1980; Henning *et al.*, 1986). This may be due to phage evolution by either mutation or recombination events or to a change in the bacterial sensitivity to phages (Jarvis, 1989).

The classification based on host range may have advantages on the applied view point i.e. selection of starter culture strain but it is of little taxonomic value as they do not relate to other classification schemes (McGrath *et al.*, 2004a). For example, it was reported that *S. thermophilus* phage with similar host ranges showed limited DNA homology whereas those with higher DNA homology exhibited a completely different lytic spectrums (Mata and Ritzenthaler, 1988).

Serology

In 1921, Bordet and Ciuca showed that blood sera of rabbits previously injected with active bacteriophage filtrates contained antibodies (antiphage) which neutralized the activity of bacteriophage. Since then serological methods have been used to classify bacteriophages (Wilkowske *et al.*, 1954). After Nichols and Hoyle (1949) first classified lactic *Streptococcus* bacteriophages into three serological groups, different attempts have been made to serologically classify *S. thermophilus* and lactococcal bacteriophages (Mata and Ritzenthaler, 1988; Brüssow *et al.*, 1994; Jarvis 1989; Kivi *et al.*, 1987). A serious drawback of this method was that it was based on differences in antigenic properties exposed on the surface of the virus which only indicated an expressed small part of the genome (McGrath *et al.*, 2004a). This disadvantage hindered the development of a sensitive detection method.

Structural protein profile

The structural protein profile has been commonly used to classify *S. thermophilus* bacteriophages and was found to generally agree with other schemes (McGrath *et al.*, 2004a). Phages from the same species have nearly the same morphology, share strong DNA homology and exhibit similar structural protein profiles (Jarvis *et al.*, 1991). While most of the small isometric headed phages have one major structural protein, the large isometric

and prolate headed phages have in common at least three major structural proteins (Moineau *et al.*, 1992; Prevots *et al.*, 1990; Braun *et al.*, 1989; Powell *et al.*, 1989). Major proteins are used to designate the protein that is in high concentration in the phage structure while minor proteins are numerous but of lower abundance. It was reported that there was a correlation between the method of DNA packaging i.e. *pac* or *cos*-type and the number, and type of structural proteins for both *S. thermophilus* and *Lactobacillus delbrueckii* phages (Le Marrec *et al.*, 1997; Forsman and Alatossava, 1991).

DNA homology

The development of an ordered taxonomy is hindered by the mosaic nature of bacteriophage genome (McGrath *et al.*, 2006). Thus classification of LAB phages based on DNA homology evaluated the entire genome for a repeated small portion (McGrath *et al.*, 2004b). This specific portion of the phage genome may encode the structural gene (McGrath *et al.*, 2004b). On the basis of DNA, DNA hybridization studies of twelve genetically distinct lactococcal phage species have been defined (Jarvis *et al.*, 1991). Based on repeated reports, all known *S. thermophilus* phages exhibit DNA homology (Quiberoni *et al.*, 2003; Brüssow and Bruttin, 1995; Benbadis *et al.*, 1990) and more closely related to one another unlike *Lactococcus lactis* phages (Le Marrec *et al.*, 1997; Fayard *et al.*, 1993; Neve *et al.*, 1989).

Origin

The origins of the phages that colonizes the dairy plants are not fully answered (Madera *et al.*, 2004). Different hypotheses have been attributed to describe the origin of bacteriophages in dairy plant. Some of them are attributed to natural widespread as dust-born infection (Whitehead 1953), the non-sterile environment inside the factory i.e. air, appliance, workers, large exposed area during cheese production (Szcepanaska *et al.*, 2007), and whey contamination (Dupont *et al.*, 2005; McIntyre *et al.*, 1991; Whitehead, 1953). The raw milk and the lysogenic starter culture are now clearly identified to be the main origin of phages in dairy plant (Fig. 1) (Szcepanaska *et al.*, 2007; Josephsen and Neve, 2004; Bruttin *et al.*, 1997; Brüssow *et al.*, 1994). Once an empty vat or a failure in fermented batch occurs, the infection with the virus is widespread throughout the dairy plant (Szcepanaska *et al.*, 2007). Their short latent period and resistance to pasteurization hinder their complete eradication from dairy plant.

Life Cycle

Bacteriophages are basically classified into virulent and temperate phage, and each has two types of life cycle, so called lytic and lysogenic. Lytic cycle is the process which a number of phages are released when lactic acid bacteria as a host cell is destroyed by their off-springs. Lysogenic cycle is the process which host cell infected

Fig. 1. The bacteriophages count (y axis) versus days (X axis) before (-ve numbers) introduction of new starter strains and after (+ve numbers), *S. thermophilus* starter strain types (Z axis) (Bruttin *et al.*, 1997).

with phage grows and divides into two host cells, and the host cell produces phages under specific condition such as UV radiation. Lytic cycle of phages are more harmful than lysogenic in dairy industry (Bush, 2002; Campbell and Reece, 2001).

Lytic cycle is started from attachment of bacteriophage on the host cell. The phage does not randomly attach to the surface of the host cell. They use specific surface structure called receptor site and use liposaccharide or protein of cell wall as their receptor. After phages attach on the host cell, they make a hole on the surface of the cell and inject their DNA into the cell. The host cell then synthesizes proteins for producing the phages at the same time. Lytic enzymes destroy rapidly the cell wall of the cell to release progeny phage after assembling phage particles (Nelson *et al.*, 2006). The process above could be one cycle of lytic bacteriophage. Since the reproduction of this cycle is rapid, the contamination of the phages in the dairy plants is vulnerable.

A lot of phages related to starter culture are tracked in lysogenic cycle. When the host cells are infected, the last

phase of their replications destroy all host cells. So, there is a risk that phages are exposed to environmental hazard for months or years. Lysogenic phage favors lysogenic cycle to avoid the above situations. Every cell infected by bacteriophage can grow and divide cell in lysogenic cycle for a long time (Prescott *et al.*, 2002). The cell infected by bacteriophage can produce off-spring phages and be destroyed under specific condition such as UV radiation. When lysogenic bacteriophages are in starter culture more than 10^6 PFU/mL, there is a decrease of acid production by lactic acid bacteria, which is critical on cheese curd formation.

Epidemiology

Three phage species such as c2, 936 and p335 represent the majority of detected phages (McGrath *et al.*, 2004a). In the reports from European countries, New Zealand, United States and Canada, 936 types of lactococcal phage were found to dominate (Fig. 2, Table 1) (Szcepańska *et al.*, 2007; Bissonnette *et al.*, 2000; Moineau, 1996). Dif-

Fig. 2. Geographical distribution of virulent lactococcal phages in dairy environment in Poland (Szcepańska *et al.*, 2007).

Table 1. *L. lactis* strain sensitivity towards isolated phages (Szcepanowska *et al.*, 2007)

Phage	Phage type	Bacterial strain															
		IBB 094	IBB 1767B	IBB 1772B	IBB 1780B	IBB 1782B	IBB 1783B	IBB 1784B	IBB 1787B	IBB 1788B	IBB 1791B	IBB 1793B	IBB 1794B	IBB 1796B	IBB 1800B	IBB 1807B	
bIBBg6/5	c2															+	
bIBB88P ₁	c2													+			
bIBB94P ₄	c2	+															
bIBB94g ₅	c2	+															
bIBB24g ₂	c2											+					
bIBB24tp ₁	936											+					
bIBB5g ₁	936											+					
bIBBEg ₁	936											+					
bIBB2a	936								+								
bIBB 3a	936								+								
bIBB 5a	936								+								
bIBB 8a	936											+					
bIBB 10a	936											+					
bIBB 40a	936											+					
bIBB 1	936				+						+						
bIBB 5	936				+						+						
bIBB 8	936				+						+						
bIBB 12	c2								+								
bIBB 14	c2							+						+			
bIBB 18	c2							+						+			
bIBB 19	c2							+						+			
bIBB 20	c2							+						+			
bIBB 22	c2							+						+			
bIBB 27	c2				+			+						+			
bIBB 27a	c2													+			
bIBB 29	936							+			+			+			
bIBB 36	c2			+													
bIBB 47	936				+												
bIBB 50	936				+											+	
bIBB 55	c2															+	
bIBB 61	c2					+											
bIBB 77	c2					+											
bIBB 89	c2		+														
bIBB 95	c2													+			

ferently in Germany, the dominating phages were c2 type while in Denmark the p335 phage was prevalent (Josephsen *et al.*, 1999). In the last 10 years, p335 type phage has been encountered with higher frequency and it was proposed that numbers of this species will be the dominant phage type in the dairy industry (Josephsen and Neve, 2004). Some specific strains of *Lactobacillus casei* and *paracasei* with probiotic activity are widely used industri-

ally for the production of yogurt and cheese. It was reported that the first phage (ϕ MLC-A) was isolated in South America for the probiotic strain (Capra *et al.*, 2006).

Detection

Phage monitoring is critically important in dairy indus-

try, especially in cheese plant (Brüssow *et al.*, 1994). Cheese making is a non-sterile process with high exposure to the plant environment. Raw or pasteurized milk is used in cheese manufacturing and many phages will resist pasteurization (Madera *et al.*, 2004). Since a large dairy plant can process more than 500 tons of milk per day, a phage problem can lead to great economic loss (Binetti *et al.*, 2005). A potential problem in a fermented batch is likely to occur if lactococcal phages exist in a population level of 10^5 to 10^7 PFU/mL in starter media or milk as this phage titer is able to retard milk acidification (Svensson and Christiansson, 1991). The correct and rapid identification of bacteriophages potentially able to attack starter cultures allow a prompt decision regarding the destination of contaminated milk (Del Rio *et al.*, 2007). The contaminated milk could be used for process that does not require starters or processes that uses starter cultures insensitive to the phage detected i.e. the milk could undergo UHT to be used as drinking milk or used for yogurt production (Del Rio *et al.*, 2007).

Phages can be detected in various ways. The traditional methods are spot test, starter activity test, indicator test (Valles, 1955), ATP measurement (Champiat *et al.*, 1988) and conductimetric analyses (Fig. 3) (Svensson, 1994). These traditional test proved to have different disadvantages; they are time consuming and the majority rely on the availability of single indicator strain which is not possible in cheese manufacture that rely on mixed strain culture (Brüssow *et al.*, 1994). Also these methods don't provide any information about the phage species causing the fermentation delay (Dupont *et al.*, 2005). Most impor-

Fig. 3. Conductance change of starter strain SMR S19 grown in reconstituted milk at 20°C in the presence of various amounts of bacteriophages (Δ) Control, ($\blacktriangle$) 1 PFU, ($\diamond$) 10 PFU, ($\blacklozenge$) 100 PFU, ($\square$) 1000 PFU, ($\blacksquare$) 10,000 PFU (Svensson, 1994).

tantly, the traditional methods applied are indirect as they measure the metabolic activity of the starter culture inhibited by the phages, so a slow metabolic activity of the starter can lead to false positive results (Moineau *et al.*, 1993). A flow cytometric method was also adopted in 2007 by Michelsen and co-workers (Fig. 4). Although the method can be performed in real time during the industrial process, it depends only upon the amount of infected cells rise independently of which type of phage has infected the culture. This doesn't allow a decision based on the results obtained to take any accurate decision i.e. the choice of the starter culture strain insensitive to the phage detected. Same disadvantages were observed for the petrifilm assay conducted in 2008 by Tseng and Hicks. The test is based upon the conventional method of phage enumeration where it measures the phage directly by its ability to lyse infected cells in a gel and so giving rise to plaques. The test is simple, as stated by the author, although many precautions for accurate results are required. Therefore, rapid, sensitive and reliable techniques are required for the detection of bacteriophages as they are related to a subsequent decision regarding the control measures and the fate of contaminated milk.

Immunological as well as molecular techniques are the most developing research until now due to their detection time, sensitivity and ability to identify the bacteriophage strains. For the immunological technique; an enzyme linked immunosorbent assay (ELISA) for the specific detection of 936, p335 and c2 phages was developed (Azaiez *et al.*, 1998; Moineau *et al.*, 1993). In these assays, antibodies against the major capsid protein for p335 and c2 phage were used but for lactococcal phage 936 species antibodies against intact phage were utilized. The detection limit was 10^7 PFU/mL. The most promising technique up till now is the use of DNA probes for the specific detection of 936, c2 and p335 phages. In 2000, Labrie and Moineau succeeded to detect the three species

Fig. 4. Flow cytometric analysis for MG1363 strain grown in GM17 with 5 mM CaCl_2 (a) before phage incubation, (b) 4 h after addition of phage, (c) mixture of a (95%) and b (5%) (Michelsen *et al.*, 2007).

in a single reaction step with a multiplex PCR. The detection limit was 10^4 - 10^7 PFU/mL. The method was applied on whey and phage lysate. Recently, a multiplex PCR was established that can detect in a single step the 3 common phages, in addition to those attacking the *Streptococcus thermophilus* and *Lactobacillus delbrueckii* (Del Rio *et al.*, 2007) (Fig. 5). The detection limit was 10^3 - 10^4 PFU/mL. It is known that a phage concentration below 10^5 PFU/mL in whey or milk is not considered a threat to fermentation (Suarez *et al.*, 2002; McIntyre *et al.*, 1991). This means that a method with a detection limit below 10^5 PFU/mL is important for the early intervention in dairy industry.

In 2008, Binetti and his team were successful to detect by the probes against a highly conserved region of the NTP-binding genes in *Lactobacillus casei/paracasei* phages (Fig. 6). The PCR technique employed was able to detect the phage in milk and cheese whey with a detection limit of 10^4 PFU/mL. Future works may focus on the possibility to design a multiplex PCR diagnostic kit that combines all the common phages with those recently detected for *L. casei/paracasei* to be the first sensitive and reliable tool that fulfill the dairy industry requirements.

Fig. 5. Multiplex PCR assay with different contaminations of phage species in the same sample. Reactions were performed with phage titres of 10^6 PFU mL⁻¹ (*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* phages spec.) *L. lactis* phages: P335 (P335 phage species), bIL170 (936 phage species), and c2 (c2 phage species). YAB represents the *L. delbrueckii* subsp. *bulgaricus* phage species and OBJ represents the *S. thermophilus* phage species. Lane 1: 100bp molecular marker; Lane 2: negative control (without phages); Lane 3: P335 plus bIL170 and c2; Lane 4: YAB plus OBJ; Lane 5: All five phages (Del Rio *et al.*, 2007).

Fig. 6. PCR for detection of *L. casei/paracasei*. Lane M, 100 bp ladder; lane 1, MRS; lane 2, milk; lane 3, yoghurt; lane 4, probiotic fermented milk; lane 5, probiotic cheese whey; lane 6, phage DNA (positive control); lane 7, negative control (Binetti *et al.*, 2008).

Control

The basic principles to control bacteriophages in cheese plants have been recognized since the early 1940s. Whitehead and Hunter (1945) reviewed on the measures that were used to control slow acid production caused by phage infection is still useful to plant managers today. Even 1945, they already knew that the possible contamination of phage in raw milk was due to tanks, cans and other facilities. But they did not know the lysogeny and the possibility of phage arising from starter cultures in raw milk. The knowledge of bacteriophage now vast. Therefore, the researches for developing effective means of eliminating them during cheese making are necessary.

Many approaches have been developed to control phage contamination in cheese plants including hygienic measures, such as chlorination of vats, avoiding aerosol generation and regular atomization of the atmosphere using sterilants (Avsaroglu and Buzrul, 2007; Whitehead, 1953). The use of disinfectants to clean bulk starter tanks and other vessels also has been proposed. The effectiveness of thermal and high-pressure at destroying bacteriophage has been clearly demonstrated (Müller *et al.*, 2005; Moroni *et al.*, 2002; Zottola and Marth, 1966). Other strategies include production of lactic acid starter in phage inhibitory media and starter culture rotation in cheese production facilities. In recent years, genetic tools have been developed and used to improve the phage resistance of lactic starter cultures. However, these genet-

ically modified microorganisms have not been approved for use as starter cultures. Besides, the acquired resistance characters are not stable and some phages have already adapted to them, making it unlikely that genetic modifications will lead to the elimination of phage problems. Basically, the methods for controlling phage problems in cheese making include as followed.

Inactivation of bacteriophage by thermal treatment

Some scientists have reported on the heat inactivation of phage. Normal pasteurization temperatures employed in the processing of milk and milk products would not reduce substantially the numbers of bacteriophages present in the milk. In heat treatment, it is important to establish a series of temperature and duration of heating relationships to ensure destruction of the bacteriophage. Too high temperature is not a target because this impairs the properties of milk for cheese-making. Some studies have been reported that heat treatment can cause undesir-

able changes (Walstra *et al.*, 1999). For examples, loss of nutrient; the cooked flavor of heated milk results from the liberation of sulfur group (Aboshama *et al.*, 1997). In UHT-heating, whey protein denaturation interrupted rennet coagulation and subsequently the curd became weaker (Schreiber *et al.*, 2000). A reduction of active phage was dependant on temperature (Müller *et al.*, 2005) (Fig. 7).

Inactivation of bacteriophage by high-pressure treatment

Inactivation of phage is proportional to both the number of phages and the pressure. Müller (2005) determined the inactivation of strains of *Lactococcus lactis* phages p001 and p008 which proved that the reduction in colony-forming units started at 200 MPa and the lactococci were completely inactivated at treatments between 400 and 800 Mpa (Fig. 8).

Some evidences proved that the effects of high pressure on bacteriophage by using electron microscopy (Fig. 9) (Moroni *et al.*, 2002). The prolate head and non-contrac-

Fig. 7. Effect of heat treatment for 0-120 min at 55, 60, 65, 70, 72, and 75°C on the phage titre of (A) phage P001 and (B) phage P008 in Ca-M17 broth (Müller *et al.*, 2005).

Fig. 8. Effect of high-pressure treatment for 0-120 min at 25°C and 300, 450, 550, and 600 Mpa on the phage titre of (A) phage P001 and (B) phage P008 in Ca-M17 broth (Müller *et al.*, 2005).

Fig. 9. Electron microscopy of phages treated with dynamic high pressure in PBS. (A) Untreated phage sample, (B) DHP treated sample showing phage with gap in the head, (C) DHP treated sample showing phage without tail and gap in empty head, and (D) DHP treated sample showing phage without tail and with empty head. Sample B, C and D were treated at 200 Mpa for 5 passes (Moroni *et al.*, 2002).

tile tail of the intact phage were shown in Fig. 9A. Fig. 9B-D shows the effect of treatment at 200 Mpa on the shape of phage. Under high pressure treatment, the coat protein of heads was denatured resulting in partial unfolding, from which genetic material was lost leading to inactivation (Fig. 9C). It was also observed that some phages lost their tail or part of it so they could not attach on host to inject genetic material.

Lopez-Frandino (1996) investigated that pressurization of milk at 300 Mpa improved coagulation characteristics, decreased the coagulation time by 19% and increased the curd firmness. In other words, the application of high pressure treatment for cheese-making is a good way not only to prevent from bacteriophage, but also to increase the cheese yield.

Inactivation of phage by combined heat and high pressure treatment

The powers of high pressure and temperature in destroying bacteriophage have been clearly proved as mentioned previously. However, in order to apply practically into dairy manufacturer, the reduction of pressure

and temperature are required to minimize undesirable effects but still use every strength point. Therefore, the combination of pressure and temperature has been proposed for this purpose (Fig. 10). In their experiments as

Fig. 10. Effect of combined heat and high-pressure-treatment for 0-120 min at 60°C/300 Mpa, 60°C/300 Mpa, 60°C/450 Mpa, 70°C/300 Mpa, 60°C/550 Mpa on the titre of phage P0008 (Müller *et al.*, 2005).

shown in Fig. 10, the inactivation increased with increasing pressure at a given time-temperature combination and with increasing temperature at a given pressure-time combination. Interestingly, phage was completely inactivated at 70°C and 450 Mpa after 30 min and 60 min at 60°C and 600 Mpa. The reduction by the combination of heat and pressure was much more than at separately each of the same temperatures and pressures. In other words, there was the complement each other between temperature and pressure to propose inhibit bacteriophage. This result demonstrated that selection of temperature and pressure is crucial for phage inactivation.

Inactivation of bacteriophage by thermal and chemical

Normally, thermal treatment is directly used for the bacteriophage control but some bacteriophages can be resistant to heat treatment. Therefore, biocides implemented along with high temperature may be useful for complete phage inactivation. Several biocides, such as sodium hypochlorite, ethanol and isopropanol, have been chosen for the phage inactivation. Buzrul reported that the lactococcal bacteriophage was not totally inactivated at 72°C for 15 min or 90°C for 5 min (Table 2). The inactivation capabilities of two alcohols (ethanol and isopropanol) on the viability of phages Φ pId6434 and Φ pI162 were investigated. Ethanol at a concentration of 75% inactivated all the phages for 1 min (Fig. 11). The similar result was also reported for isopropanol for 15 min.

Increasing Phage Resistance of Cheese Starters

According to Hill (1993), there are three resistance

Table 2. Effectiveness of heat treatments on inactivation of lactococcal bacteriophages (Buzrul *et al.*, 2007)

Bacteriophage	72°C, 15 min	90°C, 5 min
Φ pId6434	+	+
Φ pI105	+	-
Φ pI14721	+	-
Φ pId6737	+	+
Φ pId6636	+	-
Φ pIc6158	+	+
Φ pIc6154	+	+
Φ pI162	+	-
Φ pI1356	-	+
Φ pI13614	-	-

mechanisms against phage inhibition in lactic acid bacteria. The first is adsorption inhibition due to the incapability of attachment of bacteriophage to the host. The second is the resistance and modification systems which degrade phage DNA and a methylation component for protecting host DNA from digestion. The third is abortive infection where phage multiplication is delayed due to limit their spread to other cells allowing to the bacterial population to proliferate. Among these resistance mechanisms in lactic acid bacteria, abortive infection has been received considerable attentions. Bacterial conjugation techniques to transfer plasmids of phage resistance to starter strains have been demonstrated (Fig. 12, Table 3). Coffey *et al.* (1998) described the emergence and persistence of a novel phage for *Lactococcus lactis* subsp *lactis* DPC4268 by the introduction of pNP40 which encodes abortive infection genes. The bacteriophage-resistant mutant DPC4360 was intact in the presence of phage 4268 and successfully used in Cheddar cheese manufacture.

Fig. 11. Inactivation of phage Φ pId6434 (a) and Φ pI162 (b) with 10% (●), 50% (○), 75% (▼) and 100% (▽) (v/v) of isopropanol. $S(t)$ is the survival ratio, i.e., $S(t) = N(t)/N_0$. $N(t)$ and N_0 are the number of survivors after an exposure time t and initial number of phages (PFU mL^{-1}), respectively. The values are the mean of three replications. The standard deviation for the survival data are less than $0.24 \log_{10}$ (Buzrul *et al.*, 2007).

Lactococcal 936 phage Lactococcal c2 phage Lactococcal p335 phage

Fig. 12. Three main lactococcal phage species (Moineau *et al.*, 1999).

Bacteriophage Inhibition Media

Calcium was important for proliferation of several strains of *Lactococcus* bacteriophage while not required for growth of host bacteria (Shafia and Thompson, 1964; Norman and Nelson, 1953). Therefore, the bacteriophage inhibition media were needed where calcium medium was removed from the starter milk by ion exchange or by using of phosphates to sequester. Calcium medium was referred to as a “phage-resistant medium”, so called, PRM/PIM. Nevertheless, application of PRM/PIM to protect the growth of starters from bacteriophage has some limitations. Some phage could destroy starter cultures even if the calcium was absent (Sozzi, 1972). Other researcher reported that the use of phosphates in PRM/PIM caused injury during metabolism of starter cultures (Ledford and Speck, 1979). Some of problems of PIM have been reported in details (Robertson, 1993).

Some commercial phage inhibitory media have been used for bulk starter production. Some of these media can also be combined with pH control systems to overcome the drawbacks associated with PRM/PIM as mentioned above in which the pH control systems were to maintain the level of acidity in the bulk starter medium. Therefore, the damaged cells could be reduced when the pH dropped

below 5. Acid development in the growth medium could be controlled by neutralization, which made the starter culture active and increased in cell count. This has been helpful in limitation of the proliferation of phage in the cheese vat. Gaudreau *et al.* (2006) investigated the effect of pH control on the phage inactivation. The viable cells of strain NM33-7 under pH control were obtained 80 % compared just 46 % under without pH control. This was consisted with the report of Champagne *et al.* (1995). Sinkoff and Bundus (1983) created the bulk starter media for cheese-making with addition of monoammonium phosphate which improved buffering capacity to keep pH at 5 for 20 hours during fermentation process.

Minimizing Phage Concentration in Cheese Plant

Bacteriophages in raw milk proliferate during the fermentation process. Whey contains a large number of phages and may be a primary source of phage infection during cheese making. These phages may directly contaminate throughout the factory by air flow or via whey separation process and subsequently the failure would occur for intermediate and/or final products. Therefore, it is important to minimize the original source of bacteriophage in the plant. The equipment must be cleaned by rinsing with disinfection for several times. Good plant design was reported as an important factor to reduce the concentration of bacteriophage. The location of buildings, prevailing wind, factory drainage, correct process flows, the correct physical separation between raw milk and whey, starter streams, and correct people are important. It was proposed that the concentration of airborne phage infection in the surroundings of cultures could be reduced considerably by separating starter room from factory (Fig. 13) (Whitehead, 1953).

Table 3. List of patented natural phage resistance plasmids or mechanisms in *L. lactis*

Plasmids (systems)	Efficiency			Authors	Year*	Patent numbers
	936	c2	p335			
pTN1060	10 ⁻⁹	10 ⁻³	NA	Klaenhammer & Sanozky	1989	US4883756
pTR2030 (AbiA, LlaI)	10 ⁻⁹	10 ⁻¹	10 ⁻⁹	Klaenhammer <i>et al.</i>	1990	US4931396, US5139950
pCI528 (Ads)	10 ⁻⁹	10 ⁻⁹	NA	Daly & Fitzgerald	1991	EP0246909, IE66528 US5019506
pCI750 (AbiG)	10 ⁻⁹	10 ⁻²	NA			
pCI829 (AbiA)	10 ⁻⁹	10 ⁻²	NA	Josephsen <i>et al.</i>	1996	WO9625503
pFW094 (LlaAI)	10 ⁻⁵	10 ⁻⁴	NA			
pJW563 (LlaBI)	10 ⁻³	10 ⁻³	NA			
pHW393 (LlaDII)	10 ⁻⁴	10 ⁻²	10 ⁻⁶	Moineau <i>et al.</i>	1999	US05994118 US7119167B2
pSRQ900 (AbiQ)	10 ⁻⁹	10 ⁻⁹	1.0			
Chromosome (2227bp)	10 ⁻⁶	1.0	10 ⁻⁷	Moineau <i>et al.</i>	2006

Fig. 13. Example of satisfactory location of starter-preparation building isolated from factory (Whitehead, 1953).

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