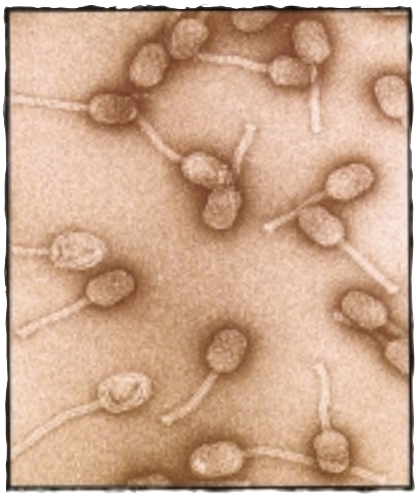
CHR. HANSEN

# Bacteriophage



***A latent problem in the cheese industry***

CHR HANSEN

**Bacteriophage – a latent problem in the cheese industry**

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Third revised edition, November 2002

*Introduction*

# Bacteriophage in modern dairy processing

*by Bryan Halfhide*

Over the years Chr. Hansen has spent considerable resources on investigating, testing, collecting and using bacteriophage from around the world. This work allows us to continue to develop strong, phage resistant lactic acid bacteria cultures for use in a huge variety of dairy applications across the globe.

Our locally based Applied Technology Laboratories (ATLs) are closely involved, on a day to day basis, in resolving phage related problems. These contacts are often of an urgent nature since phage problems are seldom detected or recognised until fairly severe. It is at this point that our dairy technologists must be able to support our customers with practical advice about reducing phage levels in the dairy and selecting the most suitable starter cultures for their particular products.

At our Research and Development centres phage are used to help in the selection of new strains, and at all stages of culture development phage are used to test the strength of new cultures. Our phage laboratory is also using traditional microbiology techniques to help increase the phage resistance of culture strains, and the results of this work have been seen in the market for many years in the form of our highly successful “pHage Control” R-700 and R-600 series cultures.

We will continue to use these techniques as tools in our development programmes whose aim is to introduce dairy starter cultures which give dairy processors reliability, consistency and control in their manufacturing operations.

This is the type of security that dairies are looking for from their suppliers. With our experience and research Chr. Hansen’s aim is to provide unsurpassed service. It is a service that requires two-way communication and faith that the recommendations we make are impartial and based solely on a wish to achieve the best possible end-product.

The objective of this booklet is to bring together, in one concise and short text, an introduction to this complex and economically important area presenting an overall view of bacteriophage and their influence on modern dairy manufacture. In preparing this booklet we have tried to look at the subject from both a technical and a practical viewpoint with papers from personnel in our Genetics Research programme and Product Development and Application department.

***Chr. Hansen’s Dairy Starter Cultures – Power in your hands***

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# Chapter 1: Bacteriophage – a general description

*by Marco Loguercio*

## 1.1. Introduction

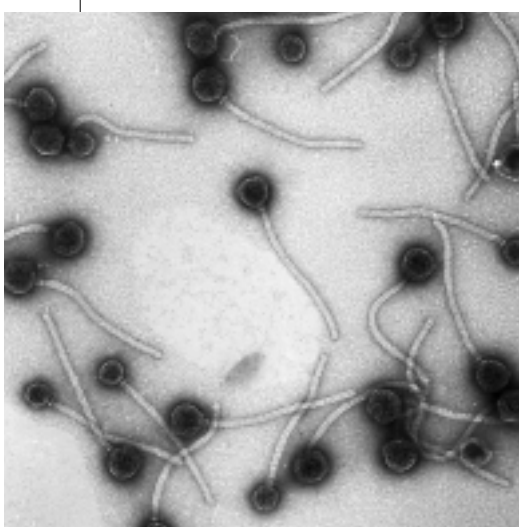
There are many factors which can inhibit the growth of lactic acid bacteria resulting in reduced acidification of cheese milk. Such factors include cleaning and disinfecting materials, liquid chemical elements, antibiotics and lysis of bacteria by bacteriophage. Bacteriophage are believed to be the most frequent inhibiting source.

Bacteriophage are present in all dairy processing environments and their action can result in increased production processing times and reduced product quality, and in the worst case complete loss of the production batch.

Bacteriophage are airborne virus particles which can attack the lactic acid bacteria used in the dairy, by adhering to the cell surface and injecting their DNA into the cell. With this mechanism bacteriophage take over the cell's metabolism, altering it to replicate new bacteriophage which are then released into the dairy environment.

Bacteriophage replicate quickly on growing cells and one phage can increase in number to 10 million phages in 5 hours under cheesemaking conditions.

Phage problems are often not identified until the damage has already taken place. It is therefore very important that possible sources of phage infection together with comprehensive measures to deal with phage attacks are known and communicated throughout the processing plant.



**Picture 1: Electron micrograph of bacteriophage of *Lactococcus lactis* subsp. *lactis*. Reproduced with permission of Horst Neve, Bundesanstalt für Milch-forschung, Kiel.**

In this chapter we will try to give a basic description of bacteriophage together with an explanation of their actions.

## 1.2. Bacteriophage

The word "bacteriophage" originates from the Greek (φαγειν = eat), the literal translation is therefore "bacteria eater" or "virus, which can infect bacteria".

In 1915 the first documented phage attack was discovered in England by Twort. In 1917 the Canadian D. Hellere discovered an element in the intestines of a sick patient which disturbed the pathogenic bacteria and named it bacteriophage. In 1928 the first bacteriophage attack against lactic acid bacteria was observed and reported.

Several years passed before science researched this phenomenon by means of comprehensive studies. In the seventies this theme was offered greater attention for the first time.

Bacteriophage are basically parasites which only develop in contact with growing bacteria. They cause no harm to human beings, animals or plants and cannot cause any human diseases.

With a size in the range of 0.1–0.2 µm phage are considerably smaller than bacteria. Therefore they can be transferred through air and can thus be present in all production areas.

Heating temperatures of 65–70°C (150–160°F) weaken the development of bacteriophage, but a complete inactivation is first reached between 90 and 95°C (194°F), 95°C (203°F), 72°C (162°F). This is of importance since cheese milk is normally pasteurised at much lower temperatures (eg 72°C for 15 seconds), and thus phage which have entered through the raw milk are not completely inactivated by the heating step.

Milk for the production of cultured milk products offers increased security because of the higher heating temperature, closed processing equipment and the fact that the final product is packed immediately after production avoiding any risk of recontamination.

Bacteriophage are widespread in nature – in the soil, surface water, outflows, plants, powder and damp places like floors, heating and cooling installations, outflow canals and areas with formation of condensed water. They are present everywhere bacteria grow.

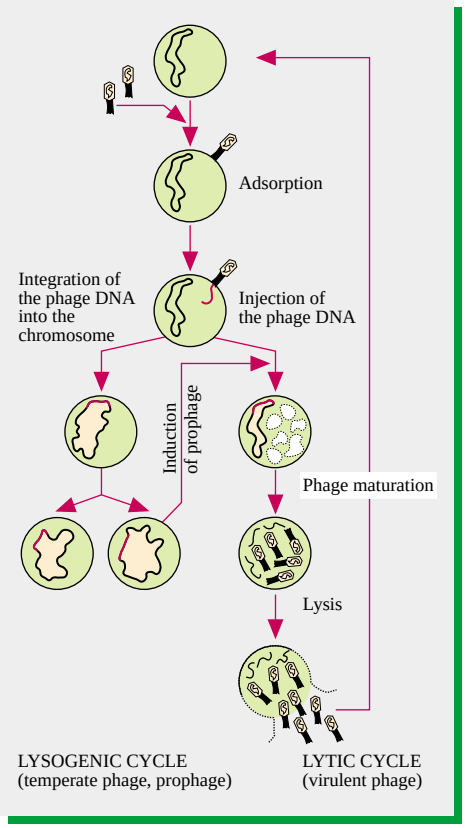



**Figure 1: Schematic representation of the propagation of virulent (right branch) and temperate (left branch) bacteriophage in a bacterial host cell (Bulletin of the IDF 263/1991)**

A bacteriophage, or phage for short, is a virus consisting of a protein head that contains nucleic acids (DNA or RNA). For reproduction bacteriophage will need to penetrate a growing bacterium as they do not have their own metabolic system and therefore cannot reproduce by themselves (characteristics of all vira).

In order for a phage to infect and penetrate a bacterium several conditions must be fulfilled, eg correct temperature range, presence of certain cell wall receptors and divalent cations ($Ca^{++}$ or $Mg^{++}$) in the medium in which the bacterium reproduces itself. Not all phages demand $Ca^{++}$ to infect bacteria; scientific studies have shown that only 50% of the examined phages demand $Ca^{++}$-ions in order to bridge the surface of the cell.

Bacteriophage which infect bacteria and are reproduced inside the cell and subsequently lyse bacteria are described as **virulent bacteriophage**. When the bacteria are lysed and new virulent phage are released, they can immediately infect new bacteria. This reproduction process of phage is described as **the lytic cycle** (Figure 1).

It can happen that the bacterium is infected by bacteriophage but the production of new phage is suppressed. In this case the phage DNA will be included in the chromosome of the host bacteria and the phage DNA sequence is now described as a **prophage**. Further reproduction of the bacteria can take place without bacteriophage being released, a process described as the **lysogenic cycle**.

A dramatic change can happen when the prophage suddenly enters the lytic cycle and the bacterium begins producing bacteriophage. Phage that are able to perform the lysogenic cycle are called **temperate phage** (Figure 1).

*A prophage can enter the lytic cycle in different ways:*

- Spontaneously
- Induced by UV-light or elevated temperature
- After treatment with the antibiotic Mitomycin C

After induction of the prophage the lytic cycle is finished and the temperate phage released after cell lysis.

## 1.3. The morphology of bacteriophage

Figure 2 shows the morphology of a typical phage. The hexagonal head is a protein envelope. Inside this envelope is the genetic information (DNA or RNA) of the phage. Under the head, the collar and the tail are located through which the DNA is injected into the bacterium. At the end of the tail, a baseplate and tail fibres are located. They will read and recognise the host specific receptors on the bacterial cell wall.

## 1.4. Steps of a phage infection

The lytic cycle starts by the phage adsorbing to the cell wall (Figure 1). This procedure is highly strain specific due to phage-receptors present on the surface of the bacterial cell wall. The receptor consists of protein and/or carbohydrate elements.

As soon as a phage is adsorbed to the cell it will inject its DNA from the hexagonal head through the tail. The phage will now take complete control of the bacterial

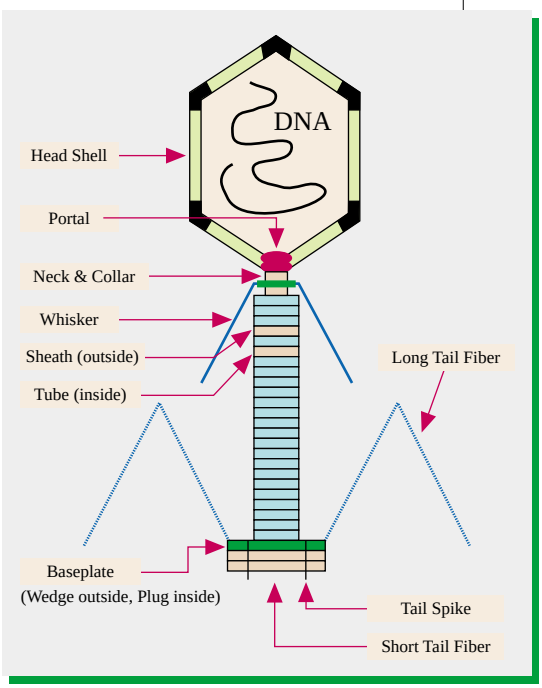
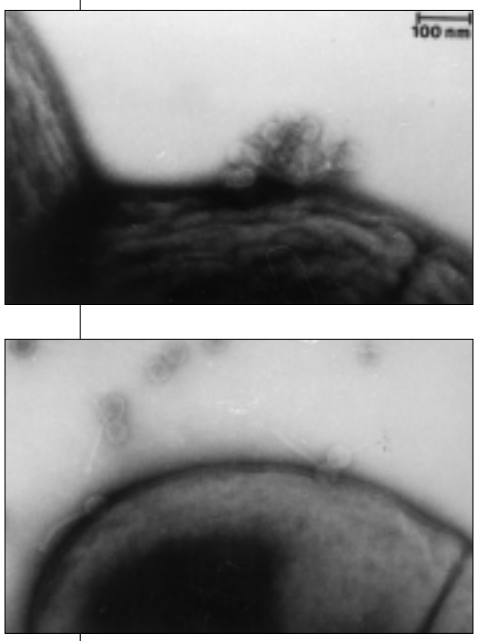



**Figure 2: Schematic drawing of a bacteriophage. Specific bacteriophage may lack individuel components. (Sherwood Casiens and Roger Hendrix)**

metabolism and initiate replication of phage-DNA and protein. New phage will be formed inside the bacterium.




**Picture 2:**
**A: Shows electron micrograph of adsorption of bacteriophage on *Lactococcus lactis* subsp. *lactis*.**
**B: Shows a resistant *Lactococcus lactis* subsp. *lactis* mutant. The bacteriophage cannot adsorb to the cell wall because of a modification or complete absence of the phage receptor.**
**Reproduced with permission of Horst Neve, Bundesanstalt für Milchforschung, Kiel.**

The phage inside the bacterium produce an enzyme called lysin which breaks down the cell wall. The bacterium lyses as the peptidoglycan of the cell wall is broken down and a new phage generation is released into the surroundings. The number of phages released from each bacterium can number between 2 and 300 (burst size). These new phages are in a position to infect new bacteria. This process takes place between 30 minutes and one hour after infection depending on the temperature.

## 1.5. Phage resistance mechanisms

During a co-evolution of bacteriophage and bacteria the latter have developed several systems to defend against bacteriophage. These phage resistance mechanisms can work at each step of the phage life cycle. In the *Lactococcus* species, these mechanisms are often located on plasmids, small DNA molecules which replicate independently of the bacterial chromosome (Figure 3). The plasmids can be transferred from cell to cell by a mechanism called conjugation. This enables the fast transfer of phage resistance mechanisms within a bacterial population.
The naturally occurring phage resistance mechanisms are summarised in Figure 4. Four major groups can be distinguished:

### *Inhibition of adsorption*

The phage is not able to adsorb to the cell wall because of a modification or complete absence of the phage receptor. The resistance mechanism of a great number of spontaneous resistant mutants is probably from this type.

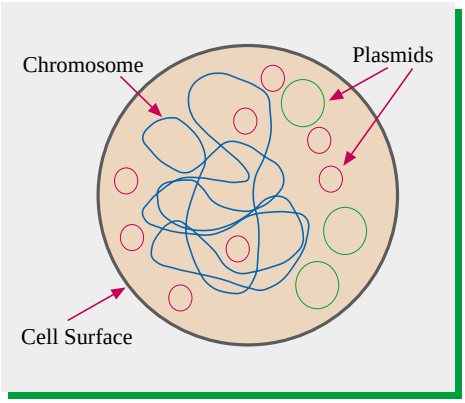



**Figure 3: The *Lactococcus* genome contains two kinds of DNA molecules, a single very large molecule called the chromosome (indicated in blue) and a number of small molecules called plasmids. Plasmids exist in multiple copies in the cell and strains often contain several different plasmids. *Lactococcus* strains naturally contain plasmids and they contain genes for a number of technologically relevant properties, for example lactose fermentation (indicated in green), and bacteriophage resistance (indicated in red).**

### *Prevention of phage DNA injection*

The bacteriophage can still adsorb to the cell wall but the injection of the DNA into the cell is inhibited.

### *Restriction and modification systems (R/M systems)*

The phage-DNA is digested after entering the cell by a specific endonuclease enzyme. To prevent the digestion of its own chromosomal DNA the second part of the R/M system, a methylase, modifies the DNA of the host organism.

### *Abortive infection*

The phage infection is aborted at a later step of the infection. The system acts within the cell but is not due to an R/M system. In the plaque assay (see page 15) one can observe a complete absence of plaques or pinpoint plaques with a lower number (a plaque is a hole in a bacterial cell lawn due to the attack of a bacteriophage). The activity often leads to cell death but no release of phage.

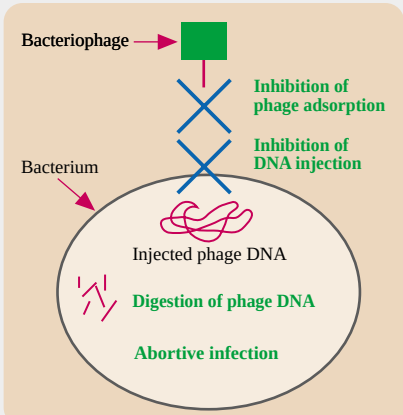



**Figure 4: Phage resistance mechanisms in lactic acid bacteria. The bacteria have evolved defense strategies which act at all steps of the phage development.**

# Chapter 2: Practical control of bacteriophage in cheese production

*by Kristian Elsborg*

## 2.1. Introduction

Bacteriophage are still the most common cause of failure in acidification during cheese production (Figure 5). The only method to secure totally phage-free environment is to sterilise equipment, milk and air. In reality this practise is not possible in cheese production but a number of precautions can prevent phage from spreading and thereby allow constant and uniform acidification of the milk used for cheese production.
The principle behind preventing phage from multiplying excessively is to break the cycle of re-contamination of the freshly pasteurised milk with phage either from the air, milk, whey residues, surfaces, pipelines or human contacts.

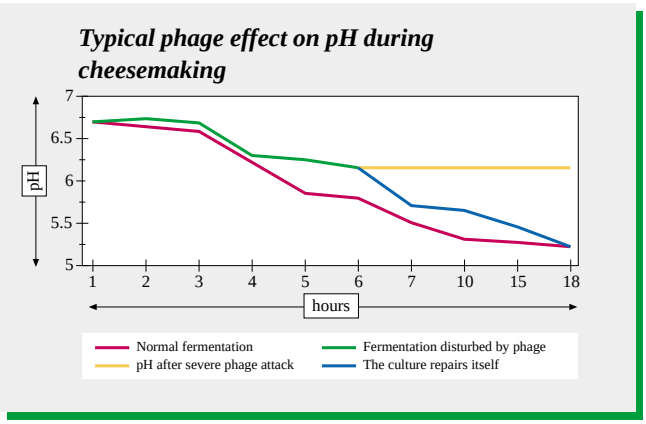



**Figure 5: Examples of the effect of different types of phage attacks. The acidification is often slowed down in the beginning of the cheesemaking process but strains not attacked will grow and take over the acidification. In situations of serious phage attacks the acidification can come to a total stop (dead vats).**

## 2.2. Production facilities and equipment design

Since bacteriophage are often airborne and can easily spread via water and aerosols it is crucial to ensure a proper design of production facilities. This will involve circulating the air away from the cheese production and in a direction following the cheese as it moves towards pressing, moulding, salting and ripening areas. A number of bacteriophage survive the normal low temperature pasteurisation and can therefore be found in the cheese milk as well as in the milk which is used for bulk starter propagation. Due to the heat resistance some bacteriophage possess and the problems connected with the inactivation of phages in the bulk starter tank headspace, it is not surprising that production of phage-free bulk starter can be difficult.
As phage are unable to multiply without a host bacterium in growth phase, the bulk starter facilities should be offered special attention. Bulk starter production should take place in separate facilities where over-pressure and sterile air filters are installed. Separate CIP systems should be used for bulk starter tanks and production equipment respectively. To secure total inactivation, also in the head space, during the propagation it is advised that pasteurisation of bulk starter media should take place at 85–90°C (185–194°F) for a minimum of 30 minutes.

**Picture 3:**
**As the production of cheese concentrates on larger and larger production sites the demands on cleaning, disinfection and robustness of the starter cultures increase.**

Whey contains lactose and bacteria and as such it is an excellent growth medium for phage multiplication. Therefore all equipment for storage of whey products should be designed in a way that unnecessary contamination of floor drains and equipment is avoided. Whey should, as a minimum, be pasteurised

(eg 72°C (162°F) / 15 secs) and transported in closed pipelines. Pasteurisation and filtering equipment should be located in separate rooms. Under no circumstances should whey be transported or stored in the same equipment as raw or pasteurised milk which themselves need to be maintained separately. Air valves on storage tanks and cheese vats should have sterile filters installed. Accumulation of phage in milk and whey in drains should be limited by frequent rinsing and disinfection.

## 2.3. Cleaning and sanitation

Correct cleaning and disinfection of equipment and tanks will limit the transfer of whey and cheese remnants from previous production batches.
Remnants should be removed between fills and cheese vats properly rinsed with water. Rinsing water should be collected and removed from the vat followed by a thorough disinfection step.
Trials have shown peroxyacetic acid to have a significant effect on the destruction of phage even at low temperatures. Further trials have demonstrated that treatment with a sodium hypochlorite solution (12.5 µg/ml) for 30 seconds removes up to $10^5$–$10^6$ phage per ml in diluted whey. If hot water is used for disinfection it is important that the temperature, measured at the outlet side, is maintained at 90°C (194°F) for not less than 5 minutes and preferably for 30 minutes.

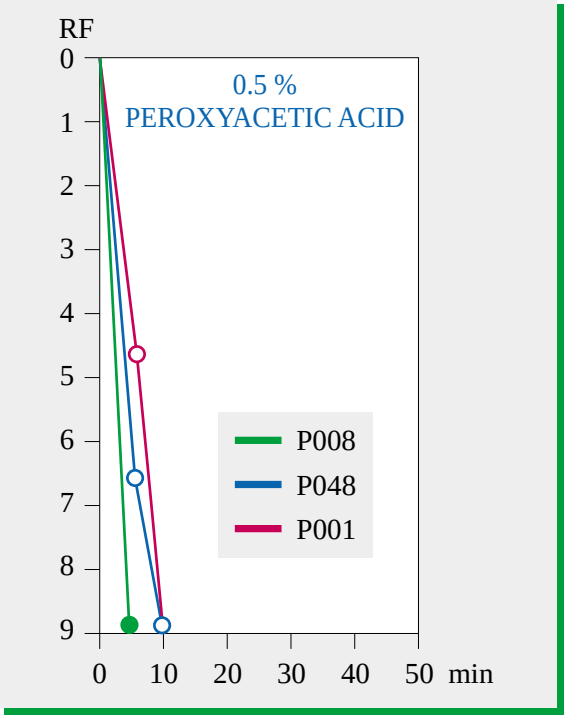



**Figure 6: Inactivation of different bacteriophage types by 0.5% peroxyacetic acid.**
**(RF = log PFU untreated/ml – log PFU disinfectant/ml).**
**Lembke and Teuber – Kiel 1981.**

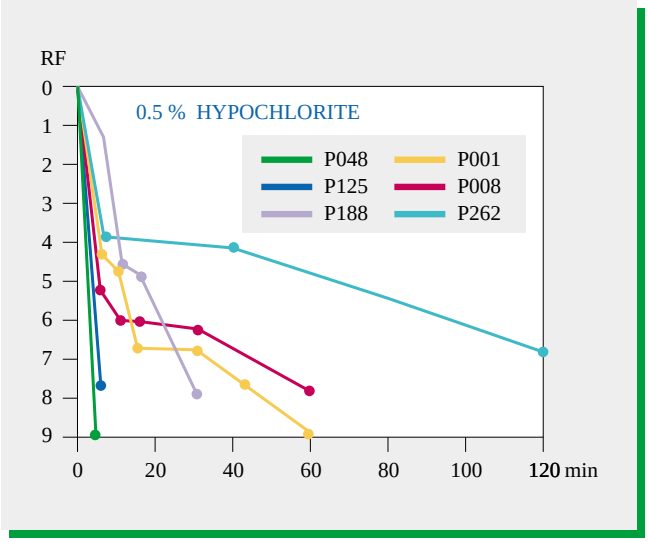



**Figure 7: Inactivation of different bacteriophage types by 0.5% hypochlorite at 20°C**
**(RF = log PFU untreated/ml – log PFU disinfectant/ml)**
**Lembke and Teuber – Kiel 1981.**

It is vital that equipment be checked for microscopic pinholes and presence of stone deposits or biofilm on a regular basis. Phage can multiply in these deposits and are difficult to inactivate during cleaning and disinfection.

## 2.4. Production

Application of DVS cultures reduces the risk of phage infection as the propagation step is eliminated. Phage insensitive starter cultures (eg phage hardened) or culture rotation systems will also reduce the risk of bacteriophage contamination.
Whey cream used for standardisation is a critical phage source and its use is not recommended. If it is used it must be heated for at least 20 minutes at 90°C (194°F). Since the coagulation process is closely followed by the concentration of the cheese curd this will limit the spread of phage, and it is advisable not to exceed a pre-ripening time of one hour.

## 2.5. Control of phage levels

To avoid potential phage attacks, continuous monitoring of phage levels is essential. Major dairy plants often collect samples, eg at the end of a week, for testing as an early warning system.
Several methods for detecting phage and their levels are available. The two most commonly used are the inhibition test and the plaque assay (spot test) (see Chapter 5).

The principle of the inhibition test is to monitor pH differences in inoculated milk. Control tubes are inoculated with starter culture and acidification is compared with tubes containing sterile filtered whey samples. A dilution series will determine the bacteriophage level.

**Picture 4: Setup of inhibition test.**

The principle of the plaque test involves mixing of phage infected whey and single strains from a starter culture in a soft agar overlay (M-17 agar) on plates. Bacteriophage will attack bacteria cells and replicate releasing more bacteriophage to attack and lyse cells in their proximity.

**Picture 5: Evaluation of plaque test. The clear zones are a result of bacteriophage which are inhibiting the growth of the host strain.**

The result will be clear areas (plaques) surrounded by opaque culture growth. The titre of the phage is expressed as pfu (plaque forming units)/ml.

Whey samples used in bacteriophage tests should be preserved prior to storage or transportation. Due to the growth of bacteria cells in the whey samples phage are able to multiply up to a thousand times in few hours thus resulting in unreliable results.

Preservation can be either through the use of sterile filtration (0.45 µm) or freezing the whey samples.

It has been demonstrated that the presence of bacteriophage in whey as high as $10^4$ per ml will not necessarily result in acidification problems during cheese production (Table 1) since bacteriophage will always be present in the production environment. The following bacteriophage levels can be used as a guide for interpretation:

| Bacteriophage per ml whey | Interpretation | Action |
|---|---|---|
| $10^2$ | **Low level.** Seldom acidification problems in production | The level is recorded |
| $10^4$ | **Medium level.** Occasional acidification problems in production | Secure optimal cleaning and disinfection |
| $10^6$ | **High level.** Acidification problems in production | Disinfect and rotate alternative culture from different phage groups (Chapter 4) |

**Table 1: Guideline to interpretation of phage levels in whey samples and suggested actions**

# Chapter 3: Development of phage resistant starter cultures

*by Thomas Janzen*

## 3.1. Introduction

Today there is no *Lactococcus* strain available which is completely resistant to all bacteriophage. Even when a new phage insensitive culture is launched on the market, it is in general possible to detect bacteriophage after a certain time. The reason for this is that phage, like bacteria, have the possibility to mutate so that new phage which are adapted to the new strains can evolve. It is possible that a phage against each strain is already present waiting for its host which was purified, propagated and concentrated by a starter culture producer and is now presented in a non sterile environment. However, the use of DVS cultures instead of bulk starter systems reduces the time bacteriophage have to adapt to the host strain and therefore leads to a higher security.

To find a remedy for bacteriophage, the isolation of strains with a good natural phage resistance is of great importance. The search for strains which combine good phage resistance with high acidification speed, flavour production etc is one of the main tasks in our laboratories. Our phage laboratory has a collection of more than 700 purified phages, most of which attack *Lactococcus* strains. These phages are used to measure the phage sensitivity of new isolated strains.

One important rule is that a new culture must be resistant to all phage from our collection when it is sold the first time.

Besides this, the culture must be **phage unrelated** to the other cultures from our product range. This means that two cultures are not attacked by the same phage or group of phages, which is a prerequisite for the substitution of a culture when a phage problem occurs.

For similar reasons the single strains in a defined mixed culture must be phage unrelated as well. If one strain is attacked by a phage then the second strain can take over the fermentation.

Possibilities to improve the phage resistance of dairy cultures are detailed in the following sections.

**Picture 6: The CH phage bank is a collection of more than 700 purified phages collected from all parts of the world. The purified phages play a major role in the development of new phage hardened strains.**

## 3.2. Isolation of spontaneous phage resistant mutants (phage hardening)

During the propagation of a phage on its host one can often observe the growth of single colonies on agar plates even though the strain is phage sensitive. These colonies are usually phage resistant mutants of the mother strain. The general opinion is that mutations within the genes coding for the phage receptor on the cell wall are often responsible for spontaneous phage insensitive strains. This could be a masking or complete absence of the receptor on the surface. Since these mutations can inactivate cell wall proteins which are important for the growth of the cell, a slow-down in acidification is often observed for these kinds of mutants. A careful examination of the isolated mutants is therefore necessary, although the use of several phage hardened strains in our cultures shows that the isolation of phage resistant mutants with a good acidification behaviour is possible.

### 3.3. Transfer of phage resistance plasmids by conjugation

Strains with a good natural phage resistance sometimes contain specific phage resistance genes. In the *Lactococcus* genus these genes are often located on plasmids and can be transferred to another strain via conjugation. Since conjugation is a natural mechanism in lactic acid bacteria, strains which are modified by transferring a phage resistance plasmid with this technique are not regarded as genetically engineered. The same is actually true for strains isolated after "phage hardening".

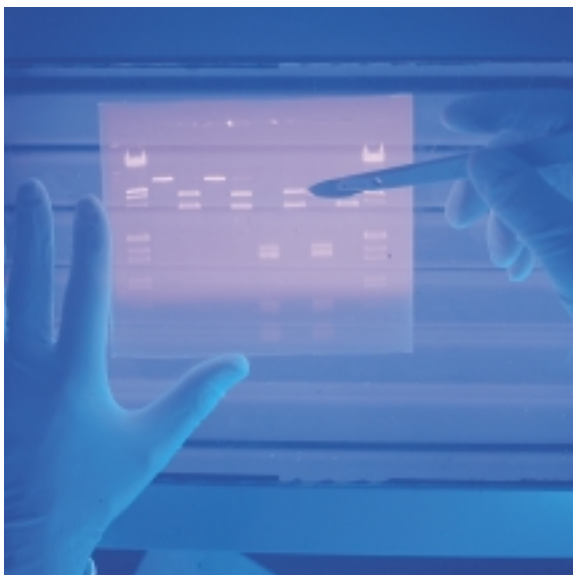
There are a number of strains in the Chr. Hansen product range which contain a phage resistance plasmid transferred by conjugation. These plasmids are responsible for resistance against a number of phages. However a single phage resistance gene does not lead to complete phage insensitivity.

### 3.4. Cloning of phage resistance genes

The use of gene technology enables the combination of several phage resistance genes in a single strain. A great number of genes coding for phage resistance mechanisms like restriction/modification or abortive infection have been identified, cloned and sequenced during the last years. In contrast to phage hardening and conjugation the directed manipulation of phage resistance is now possible. Phage resistance genes which give resistance against different phages can be combined on a plasmid and introduced into a strain which itself may have valuable acidification speed or good flavour production. Since the plasmid used as the vehicle, as well as the phage resistance genes, is originally isolated from *Lactococcus* strains, the whole system has a "food grade" character containing no foreign DNA.

Today the product range from Chr. Hansen does not contain any genetically modified strains as defined by the EU Directive 220. However considerable research is being done within this area and during the coming years we will offer our customers strains with genetically engineered phage resistance.

Chr. Hansen maintains an open and informative approach to our customers with regard to the area of gene technology and genetically modified organisms.



**Picture 7: Shows pattern of DNA bands from agarose gel electrophoresis. The DNA fragments are extracted from the gel and used in cloning experiments.**

**Picture 8: Phage team at Chr. Hansen – the team is involved in isolating phage, collecting phage information into a database, testing against new strains, validating procedures and isolating phage resistant strains.**
**From left to right: Pia Rasmussen, Jannie Schnabl, Birgitte Stuer-Lauridsen, Thomas Janzen, Dorte Overgaard Jensen.**

# Chapter 4: Chr. Hansen's DVS cultures – rotation and phage grouping

*by Kristian Elsborg*

## 4.1. Introduction

The introduction and evolution of Chr. Hansen's DVS (Direct Vat Set) concept involves the use and development of phage hardened starter cultures with a robustness that allows them to acidify milk without requiring rotation (see Chapter 3). Rotation of cultures involves some disadvantages related to changes of culture performance properties such as acidification activity, proteolysis etc. These might have an impact on the quality of the final dairy product. Furthermore there is a risk of building up an extensive phage flora against many different culture groups.

Nevertheless, specific conditions can mean that rotation of starter cultures as a preventative tool against bacteriophage becomes necessary. As the individual dairies become larger and the number of vats inoculated in one day's processing shift increases so does the phage pressure on the individual culture in production. Insufficient levels of hygiene and unfavourable design of the individual site are other factors that could make rotation necessary.

Rotation of cultures should always be followed by phage testing involving possible alternative culture solutions.

## 4.2. Development of starter culture systems

Many traditional starter cultures for cheese production have, up until the 1980's, consisted of **multiple undefined strains**. They often consist of more than 60 strains with known or unknown performance but with a certain known balance between acid producing strains and aroma producing strains. Because of the high strain diversity of these types of cultures they will normally be able to work even with a relatively large

**Picture 9: The DVS (Direct Vat Set) starter culture system offers flexibility and increased safety against bacteriophage as the period for which the culture is exposed to phage is restricted to the cheese vat (left: Frozen Direct Vat Set cultures – right: Freeze-dried Direct Vat Set cultures).**

number of phage present, sometimes with two or three cultures for back-up. The disadvantage inherent in this type of culture is that occasional attacks on some of the strains in the culture will lead to alterations in composition and thereby fluctuations in the final product quality. The risk can be diminished by the use of DVS cultures instead of bulk starters, as the period for which the DVS culture is exposed to phage present in the plant is restricted to the fermentation time in the cheese vat (4–6 hrs).

In the beginning of the 1980's Chr. Hansen released the first DVS starter cultures composed of defined single strains. Defined single strain cultures will normally consist of 1–6 strains which are well characterised with respect to acid production, salt sensitivity, temperature profiles, purity etc. Furthermore these cultures have undergone phage hardening by the classical methods. The cultures are often used in a non-rotation system, but in larger dairies with multiple fills rotation can become necessary.

## 4.3. Rotation

When the decision to rotate cultures has been made, several questions arise like: Which cultures supplement the culture in use? How often should a rotation take place etc? It is very difficult to recommend a standard guideline as the production conditions and the basis for rotation are different from site to site. As a rule of thumb rotation can take place according to two scenarios:

1. **The attacked culture is replaced by a back-up culture from a different phage group with similar performance. Based on the results from phage testing the standard culture may be re-introduced after a period of at least 3 weeks**

2. **In situations with more serious problems, a set-up with continuous rotation of 2–5 cultures and a frequency of culture replacement from between each fill to every second day could be recommended**

In the following tables the Chr. Hansen DVS cultures are grouped according to the cheese type and phage relations. A culture in rotation should not be replaced by a culture from the same phage group as science and experience show similarities in phage pattern within the same group.

Please refer to your local Chr. Hansen representative for advice on the most suitable culture for rotation.

## 4.4. Phage relations – Chr. Hansen's DVS cultures

**Phage relations – Cheddar and Feta cheese types**

| | | |
|---|---|---|
| R-603<br>R-703<br>R-604<br>R-704<br>FRC-65<br>FRC-70 | R-607<br>R-707<br>FRC-75 | R-608<br>R-708<br>FRC-60 |

*Cultures from the same column should not be used in rotation*
***FRC cultures** develop specifically for Feta cheese*

**Phage relations – Cheddar cheese types (RST/RSF blends)**

| | | | | | |
|---|---|---|---|---|---|
| RST-643<br>RST-676<br>RST-776 | RST-744<br>RSF-621 | RST-630<br>RSF-636 | RST-631 | RSF-637 | RSF-638 |

*Cultures from the same column should not be used consecutively in rotation*

**Phage relations – Dutch & Continental cheese types**

| | | |
|---|---|---|
| CH-N 11<br>CH-N 12<br>CH-N 120<br>CH-N 14<br>B-11<br>DCC-240 | Flora Danica Normal | CH-N 19<br>DCC-250<br>DCC-230 |

*Cultures from the same column should not be used consecutively in rotation*

**Phage relations – Mozzarella and Pizza cheese types (*S. thermophilus* + *Lactobaccillus* blend)**

| | | | |
|---|---|---|---|
| TCC-3<br>TCC-20 | TCC-4 | TCC-5 | TCC-6 |

*Cultures from the same column should not be used consecutively in rotation*

**Phage relations – Mozzarella and Pizza cheese types (*S. thermophilus*)**

| | | | |
|---|---|---|---|
| St-M 3 | St-M 4 | St-M 5 | St-M 6 |

*Cultures from the same column should not be used consecutively in rotation*

**Phage relations – Soft cheese types (*S. thermophilus*)**

| | | | | |
|---|---|---|---|---|
| SSC-4<br>ST-B01 | SCC-1 | SCC-2 | SCC-3 | SCC-17 |

*Cultures from the same column should not be used consecutively in rotation*

**Phage relations – Cottage cheese types**

| | | | | |
|---|---|---|---|---|
| R-603<br>R-703<br>R-604<br>R-704 | R-607<br>R-707<br>CC-02 | R-608<br>R-708<br>CC-02 | CC-04 | CC-06 |

*Cultures from the same column should not be used consecutively in rotation*

**Phage relations – Cottage cheese types (Fresco range)**

| | | | |
|---|---|---|---|
| Fresco-100 | Fresco-110 | Fresco-120 | Fresco-130 |

*Cultures from the same column should not be used consecutively in rotation*

## 4.5. Check list – Prevention of excessive phage development

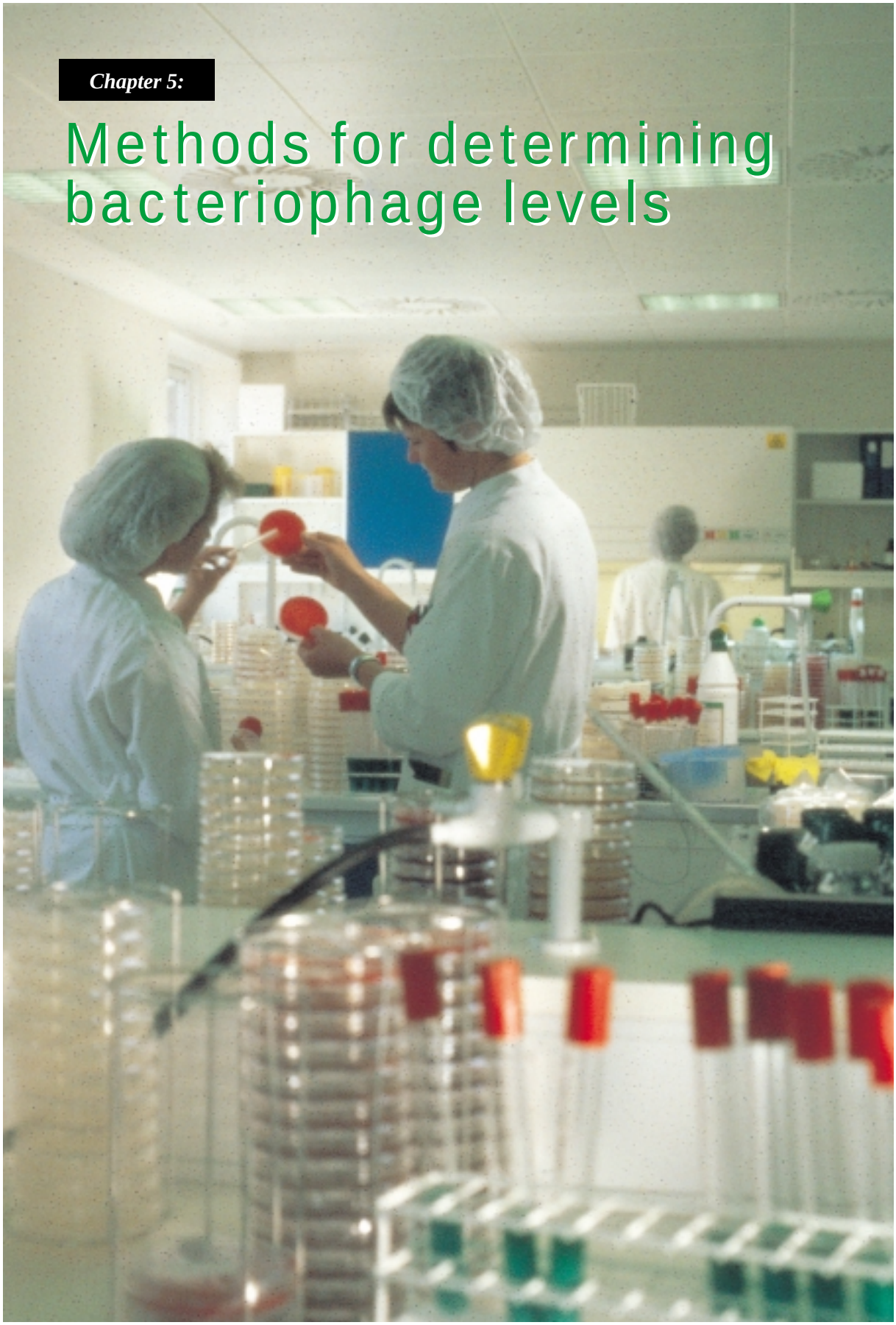
The combat of bacteriophage is an ongoing process. If the phage level in the production environment reaches a level of measurable inhibition, the actions of elimination will become more complicated. Compared to combating phage problems, it will always be less complicated and less expensive to prevent phage problems.

To be in the forefront – and thereby prevent damaging phage attacks – the check list detailed below has been made as a tool for production people in regular audits. The check list should be expanded and adapted to local procedures.

| | **Production equipment and facilities** | **Accepted** | **Corrective actions** |
|---|---|---|---|
| 1.1 | Control of pasteurisers for milk, whey and whey cream (leaks, temperature documentation, gaskets, etc) | | |
| 1.2 | Control of pumps, pipelines, seals, gaskets, leaks | | |
| 1.3 | Control of tanks (stone deposits, pin-holes, gaskets etc) | | |
| 1.4 | Floors and drains (dirt, cracks etc) | | |
| 1.5 | Water bath with disinfecting agents at entrances | | |
| **2.** | **Design** | | |
| 2.1 | Control of possible cross contamination from pipeline connections between milk and whey | | |
| 2.2 | Control of overlap from previous production in buffer tank | | |
| 2.3 | Separate location of whey handling equipment | | |
| 2.4 | Separate location of bulk starter equipment | | |
| 2.5 | Over pressure in bulk starter room | | |
| 2.6 | Heat treatment of bulk starter media | | |
| 2.7 | ”Red zones” signs for areas with low traffic | | |
| **3.** | **Cleaning and sanitation** | | |
| 3.1 | Control of strength and impurities in CIP solutions | | |
| 3.2 | Control of strength in disinfecting solutions | | |
| 3.3 | Temperatures of CIP and disinfecting solutions | | |
| 3.4 | Temperatures of CIP rinsing water | | |
| 3.5 | Impurities from milk or cheese remnants in rinsing water | | |
| 3.6 | Control of pressure in CIP pipelines during cleaning | | |
| 3.7 | Rinsing, disinfection and removal of remnants in cheese processing equipment | | |
| 3.8 | Control of CIP circulation times | | |
| **4.** | **Procedures** | | |
| 4.1 | Cleaning and sterilisation – frequency and documentation (Raw milk → Final product) | | |
| 4.2 | Procedures for corrective actions if phage testing shows positive results | | |
| 4.3 | Procedures for personal hygiene, dress codes etc | | |

*Chapter 5:*

# Methods for determining bacteriophage levels



# Inhibition test

## 1. Principle

This test indicates whether a sample contains compounds which inhibit the growth of bacteria. For this, one tube is inoculated with the culture, a second tube with the culture and the test sample. A delay in acidification shows that some inhibiting compounds are present. Since bacteriophage cause inhibition of acidification this test can be used for the detection of phage, eg in whey samples.

## 2. Application

The test is used to identify bacteriophage in liquid samples like whey or fermented milk products. It can also indicate phage in frozen or freeze-dried products if liquid solutions are prepared before the testing.
The inhibition test is particularly used with mixed cultures because in this case a plaque assay is very problematic.

## 3. Materials

- Reconstituted skim milk (RSM), 9.5%, 115°C, 15 min
- 200 ml sterile bottles
- 20 ml test tubes with caps
- Paper filter
- Centrifuge, 6000 rpm
- Pipettes, sterile, Demeter (0.1, 1 and 10 ml)
- 1/4-strength Ringer's solution
- pH-meter
- 0,45 µm sterile filter
- sterile syringes

## 4. Sampling and preparation

To avoid a propagation of phage during storage or transportation, or an inactivation of the phage, the whey must immediately be sterile filtered (0.45 µm pore size) and/or transported on ice. If dry ice is used for transportation then 15% sterile glycerol should be added to the whey sample. Normal ice might be sufficient especially if the transportation does not take long.

**Treatment of samples:**
If the whey sample has arrived at the laboratory unfiltered it is filtered through an ordinary paper filter and 5 ml is centrifuged at 6000 rpm for 15 min. If the sample is handled carefully it should be possible to remove some whey (supernatant) with sufficiently low cell count which is added directly to the test. If this is not possible then a sterile filtration (0.45 µm) might be necessary. The whey is used:

1: Undiluted
2: Diluted $1x10^{-2}$ and $1x10^{-4}$ (with 1/4-strength Ringer's solution)
3: Heat treated 90°C (194°F), 5 min.

## 5. Analysis

**Controls/cultures**
Samples can be tested against a mixed culture or the single strains.

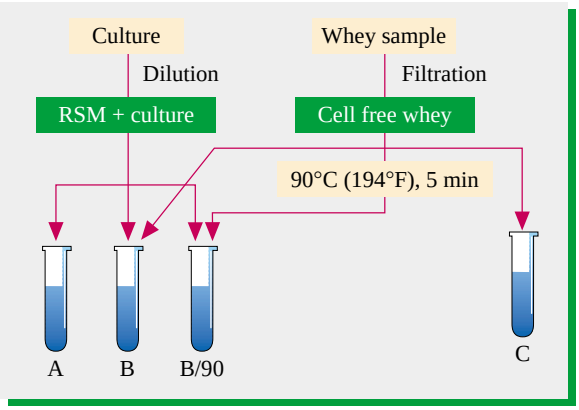
| | |
|---|---|
| **F-DVS cultures** | 1 g in 100 ml RSM, from this 1 ml is transferred to 100 ml RSM (inoculated milk) |
| **FD-DVS cultures** | 1 g in 100 ml RSM, from this 0.1 ml is transferred to 100 ml RSM (inoculated milk) |
| **Redi-Set** | 1 ml of propagated culture into 100 ml RSM (inoculated milk) |

**Test**
The inoculated milk (diluted control culture) is dispensed (10 ml) into sterile tubes:

(A) control without whey
(B) one tube for each whey sample (or more, dependent on the number of dilutions), 0.1 ml of the sample is added
(B/90) 0.1 ml of heat treated whey (90°C (194°F), 5 min)
(C) For each whey sample 10 ml uninoculated RSM (without culture) is mixed with 0.1 ml of whey to test if there are any disturbing acid producers in the whey.

The samples are mixed by gentle shaking and incubated at the temperature which was used at the dairy during the fermentation. When the pH in tube A reaches 5.5–5.0 the pH in all test tubes is measured.

**Test setup:**



## 6. Evaluation

A pH difference in tube B > 0.1 compared to tube A is considered as positive (at $10^{-2}$ dilution which resembles the undiluted whey). It does not necessarily mean that bacteriophage are present.
When the pH difference is 0.1 pH unit at a higher dilution it is likely that it is caused by a phage (see also remarks).
If the same inhibition occurs in tube B/90 it is probably caused by other inhibitors (eg antibiotics).
The whey can sometimes stimulate the growth so that the pH in tube B can be lower than the pH in tube A.
The pH in tube C should be unchanged since it is not supposed to contain acid producing strains.
To confirm that phage are present the whey should be tested with plaque test.

## 7. Safety precautions

It is very important not to spread phage from whey and other samples. Everything, which can be sterilised should be treated in boiling water or autoclaved after use.
The electrode should be rinsed in water after use and left in 70% ethanol for one hour. The laboratory bench should be wiped with 70% ethanol.
Chloramine is very effective against phage but since it can cause allergies it should be avoided.

## 8. Remarks

It is important to test at the same temperature which was used at the dairy during fermentation.
The time when the pH is measured is important as well. The pH in the control tube should be measured when pH is > 5.0 since a sample with a low inhibition might finally reach the same end pH.
Inhibition-positive samples should be sent to the Chr. Hansen laboratory in Hørsholm or to the local Chr. Hansen companies in order to verify the result (eg by plaque test).
The inhibition test shows the presence of a compound inhibiting the acidification which could have another reason than phage (especially at lower dilutions).
As well as confirming the presence of phage the laboratory will purify the phage and use them for phage typing and phage hardening of strains.
A special blank form is available which should be sent together with the positive samples (Phage Report).

# Plaque test

## 1. Principle

The plaque assay is an agar overlay method to enumerate the number of bacteriophage in a sample. The method involves mixing phage and culture in an agar overlay (top agar). Single phage will attack a bacterial cell and replicate releasing phage to attack and lyse bacteria in their proximity, resulting in clear areas (plaques) surrounded by opaque culture growth.

## 2. Application

The test is particularly used for the detection of bacteriophage in whey samples. Besides the clear identification of a contaminating phage the phage titre (phage per ml) is also indicated. It is also used to show phage sensitivity or phage resistance of a specific strain. A prerequisite is that the test strain produces a dense bacterial cell lawn on an agar plate.

## 3. Materials

i) Phage containing sample (eg whey)
ii) O/N M17 broth culture of host strain
iii) M17 agar plates, 1.5% (or an appropriate growth medium) containing 10 mM $CaCl_2$
iv) M17 top agar, 0.7% heated in boiling bath until medium is dissolved and agar melted.
Cooled to 55°C (131°F), addition of 10 mM $CaCl_2$
v) $CaCl_2$ 0.5 M stock solution
vi) Waterbath at 46°C (115°F)
vii) Boiling bath
viii) 1/4-strength Ringer's solution

## 4. Sampling and preparation

To avoid a propagation of phage during storage or transportation or an inactivation of the phage, the whey must immediately be sterile filtered (0.45 µm pore size) and/or transported on ice. If dry ice is used for transportation then 15% sterile glycerol should be added to the whey sample. Normal ice might be sufficient especially if the transportation does not take long.
If the whey sample has arrived at the laboratory unfiltered it is centrifuged at 6000 rpm for 15 min and sterile filtered (0.45 µm).

## 5. Analysis

1. Melt top agar in boiling bath and cool in 50°C (122°F) water bath

2. Carry out 10 fold serial dilutions of phage containing sample with 1/4-strength Ringer's solution

3. Take 0.1 ml from each dilution step in duplicate and transfer to separate tube.
   Add 0.1 ml of an overnight culture of the host strain grown in M17 broth.
   Add 0.1 ml from a 50 mM $CaCl_2$ solution, mix briefly and incubate for 10 min at room temperature to allow the phage to adsorb to the host cells.

4. Add 3 ml of the melted top agar 50°C (122°F), mix briefly and pour the samples evenly onto the M17 bottom agar plates, allow to set for 15 min. For control add 0.1 ml of 1/4 Ringer's solution instead of phage sample

5. Incubate the plates face up at 30°C (86°F) (for *Lactococcus*) over night

## 6. Evaluation

Examine the plates for the appearance of phage-derived uniform clear lysis zones (plaques) in the lawn of the host bacteria. Use the plates where you have between 30 and 300 plaques per plate.

## 7. Calculation

Count plaques on plates and record results. Calculate plaque forming units (pfu) per ml of original sample and record that result as well.

## 8. Safety precautions

It is very important not to spread phage from whey or other samples. Everything which can be sterilised should be treated in boiling water or autoclaved after use. The laboratory bench should be wiped with 70% ethanol.

## 9. Remarks

**Spot test:**
The spot test is a modification of the plaque test. Here the cell lawn with the host bacteria is prepared first (0.1 ml cells + 3 ml melted top agar + $CaCl_2$, allow to set for 15 min). Afterwards tenfold dilutions of the phage samples are spotted onto the surface of the top agar (10 µl per dilution). The appearance of plaques within the spotted area indicates bacteriophage.
This test is not as accurate as the plaque test but enables the testing of more samples by using less material.

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