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Bacteriophages: New Tools for Safer Food?

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Abstract: The recent FDA approval of *Listeria*-specific bacteriophage preparations for food preservation has opened the door to new applications of these natural bacterial killers. Bacteriophages are viruses that only infect and lyse bacterial cells and are harmless to mammalians. There is now a renewed interest to use the ability of these viruses to kill pathogenic bacteria for biotechnological purposes. We will describe the biology of bacteriophages, the concept of „phage therapy" and recent applications of phages as biocontrol agents with emphasis on applications for food safety.

Zusammenfassung: Die amerikanische Zulassungsbehörde FDA (Food and Drug Agency) hat kürzlich die Verwendung von Bakteriophagenpräparaten zur Bekämpfung von *Listeria monocytogenes* in Lebensmitteln erlaubt. Bakteriophagen (Phagen) sind Viren, die spezifisch Bakterien töten. Für Zellen höherer Lebewesen sind diese Viren ungefährlich. Die *Listeria*-Phagenpräparate zeigen die potentielle biotechnologische Nutzung solcher Viren, die Sicherheit von Lebensmitteln durch eine gezielte Bekämpfung eines pathogenen Keims zu erhöhen.

In dieser Übersichtsarbeit werden verschiedene Anwendungsbereiche von Phagen in der Lebensmittelproduktion vorgestellt, die sich zurzeit noch weitgehend im experimentellen Stadium befinden. Die Applikation von Phagen an lebenden Tieren („preharvest"), um die Keimzahl eines pathogenen Bakteriums zu reduzieren, ist als Phagentherapie bekannt. Ihre Anwendung am Lebensmittel („postharvest") wird als Biokontrolle bezeichnet. Der sichere Gebrauch von Phagen erfordert Kenntnisse über ihre Biologie; daraus leiten sich spezielle Anforderungen an Präparate ab, die in der Lebensmittelproduktion verwendet werden sollen.

1. Introduction

Bacteriophages (phages) were independently discovered by Twort (1915) and d'Herelle (1917). Phages are viruses that infect bacteria. They are either virulent and lyse the target bacteria or they are temperate, which means that they may be integrated into the bacterial genome. Phages are probably the most abundant life form on earth (Brüssow and Kutter, 2004) and are found in all natural environments.

After their discovery phages were used to combat bacterial infections (1920s to 1950s). Particularly the microbiologist d'Herelle used phage preparations for medical purposes first in animals and later on in humans (Summers, 2001). D'Herelle was one of the founders of the Eliava Institute of Bacteriophage, Microbiology and Virology in Tbilisi, the capital of the former Soviet republic Georgia. This institute continued to produce phage preparations for therapy and prophylaxis for well over half a century (Holzman, 1998). In the western world the use of phages for therapy was abandoned in the 1940s largely due to the discovery of antibiotics and also as a result of the ambiguous results of phage treatments (Summers, 2001). Nevertheless, phages have been used for decades in fundamental biological research in laboratories all over the world and the impact of this research on modern molecular biology has been huge.

The world wide increase of bacteria resistant to antibiotics has stimulated research to find alternative approaches to combat pathogenic bacteria. In this context a renewed interest in phages has arisen to employ their antibacterial capacity for the elimination of pathogenic bacteria.

This article aims to provide an insight into how phages may be used to produce safer food by exploiting their ability to kill pathogenic bacteria. This goal can be achieved by eliminating zoonotic bacteria in domestic animals used for food production (preharvest). The use of phages in living organisms has been designated phage therapy. The use of phages in the food production process at later stages (postharvest), e. g. their application directly to retail foods, has been termed biocontrol. In some cases phages have been used to control plant pathogens, an overview for these trials is reported elsewhere (e. g. Greer, 2005).

We will give a short overview about the basic molecular biology of phages before we will focus on the potential applications of phages in the food industry.

2. Biology of Phages

Phages are viruses which specifically infect bacteria. Phages occupy all habitats where bacteria live and it can be predicted that every bacterial species on earth (including archaebacteria) is susceptible to at least one phage. The total number of phages has been estimated to add up to 10^{31} particles (Brüssow and Kutter, 2004), ten times more than bacteria, making phage the most numerous category of „organisms" on our planet. The host range of phages is very diverse. Some phages are highly specific for single bacterial isolates while others reveal a broad host range infecting a wide range of bacterial strains of a genus.

Phages are classified into families according to their morphology and genome. Most phages possess double-stranded DNA but some have single-stranded DNA or even RNA. Depending on their life style, phages can be divided into two groups, virulent phages and temperate phages. Virulent phages strictly follow the lytic cycle whereby they multiply in the bacterial cell and lyse the cell at the end of the cycle releasing phage progeny. By contrast, temperate phages have the option to enter the lysogenic cycle. This means that the phage DNA is inserted into the bacterial chromosome where it replicates as part of the host genome for a period of time. At this stage, the phage is called prophage. The lysogenic host bacterium may carry the prophage for many generations until it may be induced to enter the lytic cycle under adverse environmental conditions. The prophage starts the lytic cycle, at the end of which phage progeny are released from the lysed host cell.

The lytic cycle, which is basically identical in virulent and temperate phages, is composed of the following steps: (1) Adsorption of the phage on the bacterial cell wall by binding to a specific receptor. (2) Injection of the phage genome into the bacterial cell. (3) Replication of the phage genome. (4) Synthesis and assembly of phage proteins involved in the formation of new capsids. (5) Encapsidation of the replicated phage genome. (6) Cell lysis and release of new phage particles (Fig. 1).

To combat pathogenic bacteria, it is a prerequisite that the selected phages are able to kill their hosts. Therefore, all steps of the lytic cycle listed above have to take place. Following this requirement, temperate phages are generally less suited for biotechnological applications, because they may lysogenize their hosts. Moreover, lysogenic bacteria are immune against superinfection with the same or a closely related phage.

Nevertheless, also virulent phages have to meet some demands, before they can be used for phage therapy or biocontrol purposes. First of all the sequence of the phage genome should be determined to find out if the phage DNA carries any genes coding for virulence factors like toxins. There are a lot of prominent toxins known whose genes are located on the genome of (temperate) phages, e. g. some botulinum toxins, cholerae toxin, diphteria toxin and shiga toxins. The next requirement pertains to the host range of the phage, which has to be defined. Certain pathogenic species or strains shall be killed by the phage while other non-pathogenic bacteria should survive the attack. The host specificity of a phage mainly depends on its receptor on the bacterial surface. For the attachment, phages can use different parts of lipopolysaccharide (LPS), capsules, flagella, fimbriae or other surface proteins. By mutations or loss of the phage receptor, bacteria may become resistant to a certain phage. This shows that the adsorption of a phage is not only the first but also an essential step in phage infection. However, bacteria may also become resistant to a phage by acquiring a restriction-modification system that degrades the injected phage DNA or by mutations, which affect proteins pivotal for the phage replication or assembly. Regardless of the mechanisms, which in individual cases lead to resistance, phages intended to be used for applied purposes should kill their host as efficient as possible.

3. Safer Food through Healthy Animals – Phage Therapy

One of the first experiments in the western world to reexamine the therapeutical value of phages were carried out in calves, piglets and lambs in the early 80s of the last century (Smith and Huggins, 1983). In this study a number of fundamental aspects of phage therapy in a natural environment of domestic animals were investigated. The results were that a mixture of two phages protected calves against a potentially lethal oral infection with an enteropathogenic strain of *Escherichia coli*. Calves that responded to phage treatment had much lower numbers of the *E. coli* strain in their alimentary tract than untreated calves. Usual-

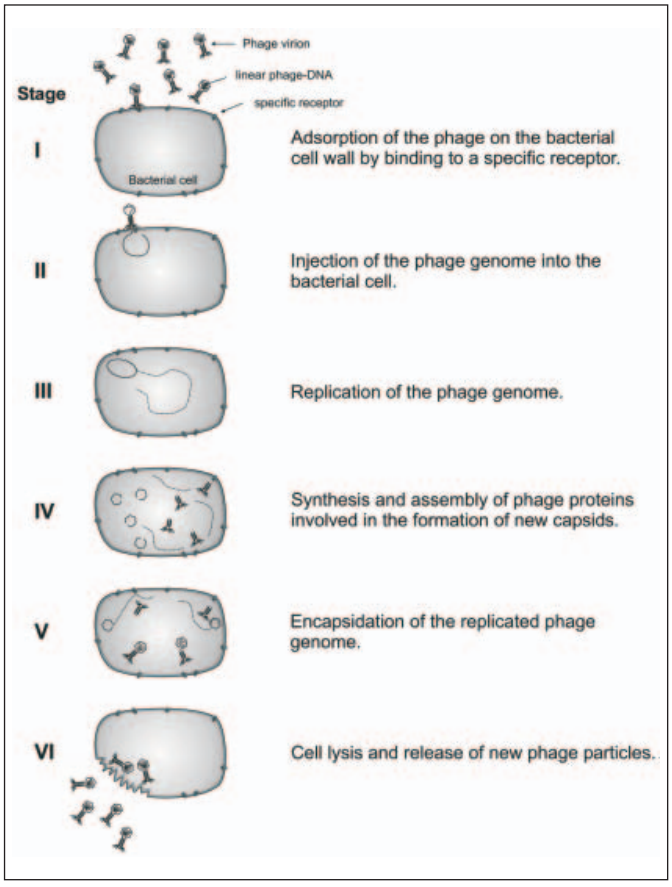

Fig. 1 Lytic cycle of bacteriophages.

ly, high numbers of phage were present in the alimentary tract of these animals. At death, most calves that had not responded to treatment with phages had high numbers of mutants of the pathogenic *E. coli* strain resistant to one of the phages in their small intestine. Phage-treated calves that survived the *E. coli* infection continued to excrete phage in their faeces. The phages survived longer than the pathogenic *E. coli* strain in faecal samples taken from phage-treated calves and exposed to the atmosphere in an unheated animal house. Calves inoculated orally with faecal samples from phage-treated calves that contained sufficient numbers of the pathogenic *E. coli* strain to cause a lethal infection remained healthy. A mixture of two phages cured diarrhoea in piglets caused by another pathogenic strain of *E. coli*. The numbers of the infecting bacteria and phages in the alimentary tract of the piglets resembled those in the calves. A similar result was obtained in lambs.

In the study of Smith and Huggins (1983) basic aspects of phage replication and spread of phages in animals in the natural environment of the stable were studied. The study revealed that the development of resistance against one phage may occur rather quickly. The use of preparations containing several phages specific for the target bacterium therefore, may be a necessary prerequisite for a successful outcome of a therapy. Furthermore, the study revealed other important aspects of the interaction of phages and their target bacteria. For example, the phages were stably maintained in the gut of the animals demonstrating that enteric phages were stable in the gut environment. In addition, it was shown that phages were able to spread through the environment thus protecting other animals against the pathogen.

In the mean time a number of other studies reporting phage applications in bacterial infections have been published and several excellent review articles are available which summarise these results (Barrow and Soothill, 1997; Duckworth and Gulig, 2002; Sulakvelidze et al., 2002; Weber-Dabrowska et al., 2003; Skurnik and Strauch, 2006). In this article we will limit our report to such studies in which phage therapy was carried out to reduce pathogenic bacteria in domestic animals with the aim to produce safer food.

Escherichia coli

Application of phages to reduce enterohemorrhagic *E. coli* O157:H7 have had mixed success (Greer, 2005). Ruminants and especially cattle and sheep are asymptomatic carriers of *E. coli* O157:H7. Some promising results have been reported recently. Phages specific for the pathogen were isolated from bovine faeces and sewage. While orally applied phages did not reduce the intestinal *E. coli* O157:H7 titer in sheep, phages administered rectally to steers reduced the number of pathogenic bacteria significantly (Sheng et al., 2006). In another study faecal shedding of *E. coli* O157:H7 showed a reduction within two days in sheep (Raya et al., 2006). However, no complete elimination of the pathogen from ruminants was achieved by phage therapy.

Salmonella

Salmonella enterica serovar Enteritidis is one of the most important salmonellae associated with chicken meat and eggs. Two phage therapy trials have been reported to reduce the concentration of the pathogen in the caeca of chicken (Sklar and Joer, 2001; Fiorentin et al., 2005). Both phage treatments reduced the average faecal counts of the inoculated *Salmonella* strains, but the pathogen was not completely eradicated. In the experiments of Fiorentin et al.(2005) the decrease of salmonellae was significant (reduction of 3.5 $\log_{10}$ after five days) using high phage doses (10^{11} plaque forming units) and lasted for several days. Both studies show that the use of phages would lead to a reduction of the pathogen entering the poultry production line (Rees and Dodd, 2006).

Campylobacter

Poultry meat is also a major source of *Campylobacter jejuni* infections. It appears that the pathogen is a natural commensal of the avian gut and is acquired from various environmental sources leading to a high prevalence in broiler chicken flocks. The analysis of chicken ceaca revealed the interesting observation that in the presence of *Campylobacter* specific phages a reduced caecal count of *Campylobacter jejuni* was found compared to samples with *Campylobacter* alone (Atterbury et al., 2005). Two phage trials have been reported in the last years. In both studies phage treatments of infected broilers reduced the faecal *Campylobacter* counts for several $\log_{10}$ units after administration, however, after few days the pathogen counts increased again (Wagenaar et al., 2005; Loc Carillo et al., 2005). In one study the prevention of infection was tested by administration the phage before the infection with *Campylobacter*. However, the colonization by *Campylobacter* was only delayed in the prevention group compared to the phage free control group (Wagenaar et al., 2005).

While none of the phage therapy trials so far led to an eradication of the target pathogen from the animals, the reduction of pathogens at the entry of the food production chain would be a sensible goal. This goal could be achieved by administering phage preparations shortly before slaughter (Tab. 1).

Tab. 1 Examples of phage applications for food safety.

Stage of food production	pathogen	trials
„preharvest“	*Escherichia coli*	sheep cattle
phage therapy	*Salmonella*	chicken
	Campylobacter	chicken
„postharvest“	*Listeria*	poultry, meat fruits
biocontrol		cheese
	Salmonella	cheese fruits poultry
	Campylobacter	chicken
	Escherichia coli	beef
	spoilage bacteria	meat

4. Biocontrol with Lytic Phages for Food Protection and Conservation

The idea that phages could be used to specifically eradicate pathogenic bacteria applies also to postharvest stages of the food production chain. In the same way as phages can be used to eradicate or prevent zoonotic pathogens from living animals, they may be employed to decontaminate carcass meat or disinfect the surfaces of ready-to-eat products. Phages have been successfully used to control pathogenic and spoilage bacteria during the postharvest storage of foods under a variety of environmental conditions.

Listeria

The first phage preparation to be used commercially contained a mixture of phages against *Listeria monocytogenes*. In the Federal Register of August 18, 2006, the US Food and Drug Administration (FDA) announced that it had approved the use of a phage preparation made from six purified phages to be used on ready-to-eat meat and poultry products as an antimicrobial agent against *L. monocytogenes*. Foodborne infections due to *L. monocytogenes* are often associated with fresh or minimally processed food. As the pathogen is an environmental bacterium contamination occurs via various routes during fermentation processing, storage, or packaging of foods. The approval of the phage preparation, developed from a company in USA, followed studies in which the effectiveness of the phage preparation against the pathogen on food was proven and a spray application in meat and poultry processing plants was permitted. The approved phage cocktail has antimicrobial activity against 170 strains of *L. monocytogenes*. Former studies had shown that the phage cocktail is also effective on fresh fruits, like apple and melon, especially in combination with the bacteriocin nisin (Leverentz et al., 2003 and 2004).

Another phage, designated P100, was developed from a Netherlands company against *L. monocytogenes* and has also a potential to be widely used in the food industry. P100 was shown to be effective against a wide range of *Listeria* strains and a bioinformatic analysis of the total genomic sequence did not reveal any similarities of P100 genes or gene products to any genes or proteins or other factors known or believed to play a role in the pathogenicity or virulence of *L. monocytogenes* (Carlton et al., 2005). As a proof of concept, the application of P100 in the ripening process of soft cheese (red-smear soft cheese) was performed. The surface of the cheese was artificially contaminated with *Listeria* and a significant reduction (at least 3.5 $\log_{10}$ units) or complete eradication of *Listeria* viable counts were achieved. Listex P100 bacteriophage product received also approval from the FDA for use on cheese. The approval was granted under the FDA's GRAS (Generally Recognised As Safe) procedure for use on cheese.

Salmonella

Studies on the biocontrol of *Salmonella* in different foods have also been promising. *Salmonella* Enteritidis was inoculated into cheddar cheese and phages were added at a fixed ratio (Modi et al., 2001). While in untreated controls the number of pathogens increased, the presence of phages decreased the counts. In cheese made from pasteurized milk salmonellae became undetectable after a long storage, while all cheeses without phage contained viable pathogens. Given that naturally contaminated cheese would have a lower level of salmonellae than the experimentally contaminated cheese, the potential of the phage as an effective control in cheese production is promising (Rees and Dodd, 2006). Leverentz et al. (2001) studied the biocontrol of *Salmonella* Enteritidis on melon slices and artificially wounded apple at different storage tempertures (5 °C–20 °C) using a cocktail of four lytic phages. Probably due to pH differences a decline of the pathogen on melon was found but not on apple. Another biocontrol study was published using a broad host range phage (Felix O1) and a variant of this phage infecting *Salmonella* Typhimurium DT104 (Whichard et al., 2003). The experiments were carried out on chicken frankfurters and revealed that under certain conditions (temperature, inoculum) a reduction of salmonellae could be achieved.

Campylobacter/Salmonella

In experiments that were carried out in parallel the reduction of *Salmonella* Enteritides and *Campylobacter jejuni* on chicken skin by application of lytic phages was studied (Goode et al., 2003). The reduction of *Salmonella* was increased by raising the number of phages per pathogen. At very high ratios of phages to host cell (MOI = multiplicity of infection of 100 to 1,000) the recoverable salmonellae were reduced by 2 $\log_{10}$ units over 48 h. Using even higher MOI it was possible to eliminate other *Salmonella* strains – possibly by a simple lysis mechanism (lysis from without). This means that very high phage MOI might be used for eliminating pathogens without the necessity that the bacterium grows and replicates during the phage application. In case of *Campylobacter* the same observation was made: At 4 °C, a temperature at which *Campylobacter* does not grow the phage treatment reduced the counts of the pathogen significantly. When chicken skin was incubated at ambient temperatures (18 °C–22 °C) a reduction of *Campylobacter* of more than 95 % was found at a multiplicity of infection of around 100.

Escherichia coli

The evaluation of a cocktail of three phages for biocontrol of *Escherichia coli* O157:H7 on beef was reported from O'Flynn et al. (2004). Application of the phage in broth cultures reduced the cell numbers of *E. coli* for 5 $\log_{10}$ units. Low cell numbers of the pathogen were inoculated on beef surfaces and after incubation at 37 °C for one hour with the phage cocktail 7 out of 9 samples were free of *E. coli*. The two remaining samples had a very low bacterial count, however, given the high efficacy of the phage cocktail in broth cultures the result was a bit unexpected. These experiments illustrate that food matrices change the conditions for phage applications and that culture broth studies are of limited use for phage applications in food matrices (Rees and Dodd, 2006).

Spoilage bacteria

Another interesting use for phages in the food industry might be the increase of storage time of food through the control of spoilage bacteria. Phages have been tested on *Pseudomonas*

isolates from meat, but the narrow host range of the phages limited the use of such phages (see references in Hudson et al., 2006; Greer, 2006). Extension of shelf life from 4 to 8 days could be achieved by using phages against the spoilage bacterium *Brocothrix thermosphacta* on pork adipose tissue, however, the experimental conditions of these experiments using sterile tissue with one single bacterial isolate do not allow conclusions for a use under commercial conditions (Greer and Dilts, 2002).

5. Concluding Remarks

The application of phages to reduce pathogenic bacteria with the aim to produce safer food is so far mostly still at an experimental stage. It seems likely that phage treatments in the preharvest stage do not eradicate the pathogen but reduce the number of pathogens. Therefore, it seems likely that phage treatments should be carried out shortly before slaughter. This would mean that phages or phage cocktails would have to follow more or less the same regulatory issues like phage preparations directly applied to food.

There are a number of safety issues regarding the safe use of phages in food that will have to be considered for companies before permission for a commercial use will be granted. We will focus here on safety issues that are specific for phage preparations rather than for food additives in general.

- Phages are sometimes highly specific for their target bacteria. This may lead to the situation that a phage has to be grown on its pathogenic host. Such preparations have to be tested for the absence of the pathogen after purification. It must also be excluded that toxins or virulence factors are present in phage preparations.
- Resistance of target bacteria against phage preparations may arise. This will be more significant in preparations containing only few phages or even a single phage. Regardless of the number of individual phages present in a preparation a monitoring system for development of resistance would be sensible to ensure the effectiveness of the preparation.
- Phages may carry virulence genes thus increasing the pathogenicity of a target bacterium. It is conceivable to demand the absence of virulence genes by sequencing the genome of the phages which are present in a commercially used preparation.
- Phages may require a certain threshold level of target bacteria to efficiently lyse a population. It should be clarified which pathogen concentrations are required for efficient phage application.
- Virulent phages may themselves mutate to temperate variants and lysogenize a pathogen. Such phages may then have the capacity to move genetic material by horizontal gene transfer. A bioinformatic analysis of the phage genome should address this question.
- The microflora of a food will be influenced by the presence of phages on its surface. Changes in the composition of the microflora of a food should be intensively analysed.

Given all the obstacles to introduce a "new" antimicrobial strategy in the food industry one should not forget that phages are found in all natural environments. Phages are measurable components of the natural microflora in the food production. They are detectable from the farm to the retail outlet and are remarkably stable in all environments (Greer, 2005). Phages have been isolated from a number of foods, like e. g. lettuce, rabs, pork, oysters, mussels mushrooms, turkey, chicken, cheese, yoghurt, buttermilk and beef (references in Hudson et al., 2005).

Thus the consumer perception of adding viruses to foods will be probably very critical, however, phages are and will be present forever in human nutrition.

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