Prevalence of bacteriophages infecting *Staphylococcus aureus* in dairy samples and their potential as biocontrol agents

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ABSTRACT

The prevalence of bacteriophages infecting *Staphylococcus aureus* in dairy samples was assessed. Fourteen *Staph. aureus* strains were used in enrichment cultures of 75 dairy samples. All samples grew specific *Staph. aureus* bacteriophages. According to the host range, 8 different phages were isolated. Three of them, phages Φ H5, Φ G7, and Φ A72, were found in 89% of the samples; all the isolated phages were temperate. Phages Φ H5 and Φ A72 were used in preliminary bacterial challenge tests against *Staph. aureus* in milk. A phage mixture (1:1) was more effective than each single phage, most likely by preventing the survival of lysogenized cells. Phages inhibited *Staph. aureus* in UHT and pasteurized whole-fat milk. However, the phages were less active in semi-skimmed raw milk and little inhibition was achieved in whole, raw milk. Killing of *Staph. aureus* was observed at room temperature and at 37°C, but not at refrigeration temperature.

Key words: bacteriophage, dairy product, *Staphylococcus aureus*, biocontrol

INTRODUCTION

Staphylococcus aureus is a relevant pathogen to the food processing industry because of the ability of some strains to produce heat-stable enterotoxins and other virulence factors that cause staphylococcal food poisoning (Dinges et al., 2000; Le Loir et al., 2003). In France, for instance, 25 out of 149 foodborne staphylococcal outbreaks that occurred in 1999 were attributed to the consumption of raw milk cheeses, and 3 out of 13 were also reported in Italy (WHO, 2000). A mass outbreak of staphylococcal poisoning was reported in Japan caused by the consumption of reconstituted skimmed milk (Ikeda et al., 2005).

Staphylococcus aureus is also a frequent cause of IMI in dairy cows (Gruet et al., 2001) and may consequently contaminate milk. Mastitis caused by *Staph. aureus* is a major concern because of its resistance to antibiotic treatment and its propensity to recur (Makovec and Ruegg, 2003). Growing concerns about antibiotic resistance have stimulated research into alternative treatment methods (Skurnik and Strauch, 2006).

Bacteriophages (viruses of bacteria) were investigated as antibacterial agents as far back as the 1920s as a means of eliminating bacteria, including staphylococci, in human infections. These efforts resulted in a wide range of phage therapy research results, which have been comprehensively reviewed (Kutter and Sulakvelidze, 2005).

Bacteriophages have been also used as bactericidal agents in foods (Hudson et al., 2005). The FDA has recently approved the use of *Listeria monocytogenes* phage, Listex P100 (EBI Food Safety, Wageningen, the Netherlands) as GRAS (generally recognized as safe) for all food products (FDA, 2006). In milk and dairy products, phages have been successfully applied to prevent *Salmonella* Enteritidis development during Cheddar cheese manufacture and storage (Modi et al., 2001), *Listeria monocytogenes* growth in red smear cheeses (Carlton et al., 2005), and *Staph. aureus* proliferation in curd manufacturing processes (García et al., 2007). Therefore, there is clearly renewed interest in the exploitation of phages as antibacterial agents, with many pathogenic bacteria being targeted (Hagens and Loessner, 2007; Hanlon, 2007).

The objective of this study was to isolate a representative collection of bacteriophages of dairy origin infecting *Staph. aureus* as a preliminary approach to develop phage-based antimicrobial strategies with future applications in food biopreservation. With this in mind, we have used bovine *Staph. aureus* strains isolated from mastitic milk samples as hosts to assess the prevalence of *Staph. aureus* phages in the dairy environment. Finally, preliminary assays were performed to test the effectiveness of a phage mixture to inhibit *Staph. aureus* growth in milk.

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Table 1. Random amplification of polymorphism DNA (RAPD) profile of isolated bovine *Staphylococcus aureus* strains and efficiency of plaque formation (EOP) of phages Φ H5 and Φ A72

<i>Staph. aureus</i> strain	RAPD profile ¹	EOP	
		Φ H5	Φ A72
Sa1	A	0.72 $\pm$ 0.03	1.01 $\pm$ 0.07
Sa2	A	0	0.39 $\pm$ 0.02
Sa3	B	0	1.02 $\pm$ 0.18
Sa4	A	0	1.32 $\pm$ 0.06
Sa5	C	0	0
Sa8	C	0	0
Sa9	D	1 $\pm$ 0.05	1 $\pm$ 0.1
Sa10	E	0	0
Sa11	E	0	0.26 $\pm$ 0.05
Sa12	E	0	0
Sa13	E	0	0
Sa14	E	0	0
Sa15	E	0	0
Sa16	E	0	0

¹Strains within a RAPD group shared more than 90% similarity.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bovine *Staph. aureus* strains used for bacteriophage isolation and determination of host range were previously isolated from milk samples of mastitic cows belonging to different farms (Table 1). Eight *Staph. aureus* isolates from conventional bulk tank and organic milk (laboratory collection) were also used for phage host range determination (Table 2). Staphylococcal cells were cultured in 2xYT broth (Sambrook et al., 1989) using routine methods.

Staphylococcal Strain Typing

Bovine *Staph. aureus* strains were typed by random amplification of polymorphic DNA (RAPD)-PCR using the oligonucleotide RAPD5 from the RAPD Analy-

sis Primer Set (Amersham Biosciences Europe GmbH, Madrid, Spain). Amplification conditions were 5 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 32°C, 2 min at 72°C, and a final 10-min extension step at 72°C. The RAPD-PCR band patterns were scanned with the Gel Doc 2000 Gel Documentation System equipped with Quantity One software (BioRad Laboratories, Hercules, CA). Sorensen's similarity coefficient was calculated as a function of the presence/absence of the different bands for each pattern, different patterns being grouped using the unweighted pair group method with arithmetic averages (Priest and Austin, 1995).

Bacteriophage Enrichment and Isolation

Bulk tank milk from 72 collaborative farms in the Principado de Asturias (northern Spain), and 3 traditional cheeses manufactured in 3 different factories were used for bacteriophage screening purposes. Each milk (100 μ L) and cheese sample (500 mg) was added to 2 mL of a bovine *Staph. aureus* strain (Table 2) growing in 2xYT containing 10 mg/L CaCl₂ and 10 mg/L MgSO₄. The cultures were incubated overnight at 37°C with shaking. Samples were centrifuged at 13,000 $\times g$ for 5 min and filtered. The supernatants were subjected to plaque assays using each of the 14 strains as indicators. Plaques were reisolated, propagated, and stored at -80°C in SM buffer (20 mg/L Tris HCl, 10 mg/L MgSO₄, 10 mg/L CaCl₂, 100 mg/L NaCl, pH 7.5) containing 50% glycerol (vol/vol). Phages were purified by ultracentrifugation (100,000 $\times g$ for 90 min) followed by CsCl continuous gradient centrifugation (Sambrook et al., 1989).

Bacteriophage Host Range

The host range of phages was determined by the plaque assay: a 0.1-mL volume of stationary-phase host

Table 2. *Staphylococcus aureus* bacteriophages selected in this study along with their respective source and host range

Phage	Temperate	Source	No. of samples ¹ (n = 75)	Host range ²	
				Mastitic strains	Milk strains
Φ C1	+	Cabrales cheese	3	<u>Sa1</u>	GDC6
Φ P1	+	Peñamellera cheese	1	<u>Sa9</u>	—
Φ L7	+	Milk	1	<u>Sa3</u>	AC9, FG1, AC11, GDC6, GRA16
Φ L13	+	Milk	1	Sa1, Sa2, <u>Sa4</u> , Sa9	AC9, DC6
Φ A8	+	Milk	2	Sa1, <u>Sa2</u> , Sa9, Sa11	—
Φ H5	+	Milk	26	<u>Sa1</u> , Sa9	AC9, FG1, AC11, GDC6, GRA16, JFL2,
Φ G7	+	Milk	20	Sa1, Sa9, <u>Sa11</u>	—
Φ A72	+	Milk	21	Sa1, Sa2, Sa3, Sa4, <u>Sa9</u> , Sa11	AC9, AFG1, AC11, GDC3, GDC6, GRA16, JFL2, JFL6

¹Number of samples in which each phage was isolated.

²*Staph. aureus* strains used in the enrichment cultures to isolate each phage are underlined; — indicates that none of the strains was infected by the given phage.

culture (10^9 cfu/mL) was mixed with several dilutions of individual phage suspensions in 3 mL of molten 2xYT top agar (0.7% agar) and the mixture was poured on 2xYT agar plates. Efficiency of plaque formation (EOP) of selected phages was determined by dividing the phage titer on the test strain by the phage titer on the reference strain *Staph. aureus* Sa9. This strain was selected because it is infected by most of the isolated phages (see Results section, Table 2).

Temperate Versus Lytic Phage Determination

To determine if a phage was temperate, putative lysogens (resistant to infection) were isolated from lysis plaques. For each phage, several plaques were scratched and viable cells were colony-isolated on 2xYT agar plates. Twenty isolated colonies were challenged by the plaque assay with the corresponding phage to confirm resistance to infection. Additionally, exponentially growing cultures were subsequently induced by adding mitomycin C (0.5 μ g/mL) to confirm prophage release. After incubation at 37°C for 4 h with shaking, supernatants were filtered and tested by the plaque assay against all the staphylococcal strains to verify the host range of the phage.

Cross-Immunity Assays

Staphylococcus aureus Sa9 was lysogenized by phages Φ H5 or Φ A72 as described (see previous section). Once it was confirmed that the phage was integrated in the chromosome, cross-immunity was assessed by the spot test. Plates were prepared by adding 100 μ L of an overnight culture of the lysogenic strain to 5 mL of molten 2xYT agar (0.7%). A 3- μ L volume of the phage stock (10^9 pfu/mL) was spotted onto the surface. Plates were incubated at 37°C for 18 h and checked for clearing zones.

Phage Temperature Stability

Phage stocks were diluted in UHT whole-fat milk to obtain 10^5 and 10^7 pfu/mL. The suspensions were incubated at 4, 22, and 37°C for 8 h and the phage titer was determined. Similarly, phage suspensions were incubated at 72°C and samples were removed at 15 s, and 1, 3, 5, and 15 min for phage titration.

Phage Antimicrobial Activity in Milk

The effect of phage infection on *Staph. aureus* growth was tested in commercial UHT and pasteurized whole-fat milk, and in whole-fat and semi-skimmed raw milks (which were centrifuged at $6,000 \times g$ for 20 min to re-

move part of the fat) supplied by a collaborating farm. Milk was inoculated with diluted overnight cultures of *Staph. aureus* Sa9 (10^2 cfu/mL) and the phages Φ H5 and Φ A72 (10^4 to 10^5 pfu/mL). The mixtures were incubated at 37°C without shaking. In experiments simulating a breakdown in refrigerated storage, UHT whole-fat milk was inoculated with diluted overnight cultures of *Staph. aureus* Sa9 (10^2 cfu/mL) and a mixture of phages Φ H5 and Φ A72 (10^1 , 10^2 , and 10^4 pfu/mL). The mixtures were incubated at 4°C for 18 h and then shifted to 22°C for the following 30 h. In all experiments, samples were taken at different time intervals and scored for *Staph. aureus* on Chapman agar plates (Scharlau Chemie S.A., Barcelona, Spain) and for phages using the plaque assay. The absence of *Staph. aureus* in noninoculated milk was verified by direct plating.

Statistical Analysis

Results were compared by one-way ANOVA analysis using the SPSS 11.0 software for Windows (SPSS Inc., Chicago, IL). Experiments were performed in triplicate.

RESULTS AND DISCUSSION

Bacteriophage Prevalence in Dairy Samples

We proceeded to isolate, from the dairy environment, new staphylococcal phages that might be better adapted and, therefore, more effective for biocontrol purposes in milk and dairy products. Fourteen *Staph. aureus* strains previously isolated from mastitic milk samples and RAPD-typed were used as hosts in enrichments assays to detect phages able to infect *Staph. aureus* in dairy samples. These strains were grouped into 5 clusters (A to E) with a similarity >90% within a cluster (Figure 1, Table 1). The enrichment procedure was a very efficient method for phage isolation, and phages were detected in all the dairy samples tested (72 raw milk samples and 3 cheeses). At this step, only phages able to infect the strain used in enrichment cultures were selected for further assays. In this way, any potential prophage released from the staphylococcal strains used as hosts was discarded. It is important to note that different host ranges were revealed when phages were plated on several strains of mastitic and dairy origin, but no correlation to the RAPD grouping was observed (i.e., strains belonging to the same RAPD profile showed a distinct phage susceptibility pattern). According to the host range, 8 different phages (2 from artisan cheeses and 6 from raw milk) were obtained (Table 2). A few phages were predominant in the dairy environment studied. In our screening, 89% of the samples contained

Figure 1. Random amplification of polymorphic DNA (RAPD)-PCR profiles of bovine *Staphylococcus aureus* isolates obtained with the oligonucleotide RAPD5 and the similarity dendrogram generated by the unweighted pair group method with arithmetic averages.

one of the Φ H5, Φ G7, or Φ A72 phages, which were detected in 26, 21, and 20 dairy samples, respectively. Some of the phages were very specific, infecting only one of the panel strains, whereas others, such as Φ A72, infected a much wider range that included both mastitic and milk-derived staphylococcal strains (Table 2). The complete set of isolated phages was able to infect all our mastitic strains, and a suitable mixture of these phages would, theoretically, inhibit the major clonal staphylococcal types associated with mastitis in our region and, consequently, the main source of staphylococcal contamination in milk.

However, none of the selected phages was able to generate plaques on any of 13 *Staph. aureus* from human clinical samples (data not shown), suggesting that the livestock and human strains could be differentiated by typing with these phages. Similar results were obtained with phages CS1 and DW2 isolated from farmyard slurry (O'Flaherty et al., 2005b). Thus, the origin of phages should also be taken into account when designing either a biopreservative or a therapeutic preparation for humans.

All these newly isolated phages were temperate, because lysogenic bacteria were easily isolated from the clearing zones (Table 2). This seems to be a general feature within phages infecting *Staph. aureus* (Kwan et al., 2005). Temperate phages may be involved in lysogenic conversion and horizontal transfer of undesirable traits. Thus, their genetic content, their transduc-

tion ability, as well as their putative contribution to host fitness should be carefully analyzed before their use as biocontrol agents in food. However, because of the genetic amenability of phages, undesirable traits could be changed or deleted. Based on this, their lysogenic nature per se should not immediately preclude their potential use as antimicrobials, as reviewed by Matsuzaki et al. (2005) and Mann (2008).

Two phages, Φ H5 and Φ A72, were selected to assess their antimicrobial potential in milk on the basis of their broad host range, which comprised most of the mastitic and dairy strains (Table 2). The morphology of their virions under electron microscopy showed isometric capsids and long noncontractile flexible tails typical of the Siphoviridae family as described recently (García et al., 2007). Their EOP, where the efficiency of strain *Staph. aureus* Sa9 is taken to be 1.0, ranged from 0 to 1.32 (Table 1).

Temperature Stability of Phages Φ H5 and Φ A72 in Milk

The stability in milk of these phages was tested at different temperatures. Similar phage titers were maintained at 4°C for 8 h, confirming that 0 to 5°C is the most suitable range for phage stability (Civerolo, 1990). Incubations at 22 and 37°C resulted in 20 to 30% phage inactivation. Both phages withstood very short exposures at higher temperatures (72°C, 15 s) but the

Table 3. Survival of Φ H5 and Φ A72 phage suspensions in UHT whole-fat milk during storage at different temperatures and heat treatment

Treatment ¹	Survival ² (%)			
	Φ H5		Φ A72	
	4×10^5 pfu/mL	3×10^7 pfu/mL	4×10^5 pfu/mL	3×10^7 pfu/mL
4°C, 8 h	100	100	100	100
22°C, 8 h	78	79	72	80
37°C, 8 h	73	75	70	78
72°C, 15 s	86	98	93	99
72°C, 1 min	0	0	0	0
72°C, 3 min	0	0	0	0
72°C, 5 min	0	0	0	0
72°C, 15 min	0	0	0	0

¹Two initial phage concentrations (4×10^5 and 3×10^7 pfu/mL) were used.

²Survival was defined as pfu/mL after the treatment divided by the initial pfu/mL.

titer was reduced to below the limit of detection (<10 pfu/mL) after 1 min of exposure (Table 3). It would be expected, therefore, that mixtures of these phages will not be fully inactivated by current pasteurization processes and then might inhibit *Staph. aureus* growth during manufacture of dairy products. Resistance of phages to pasteurization has already been described (Madera et al., 2004).

Absence of Cross-Immunity Between Φ H5 and Φ A72

The ability of phages to lysogenize might hinder the use of phages as biocontrol agents. Lysogenized strains, whereby the resident repressor blocks the replication of the infecting phage, become immune to infection by similar phages (Ladero et al., 1998). However, the use of a complex mixture of several phages would minimize the risk. To test this, lysogenic cultures to each phage were generated (*Staph. aureus* Sa9 lysH5 and *Staph. aureus* Sa9 lysA72, respectively). *Staphylococcus aureus* Sa9 lysH5 was resistant to infection by phage Φ H5 but was infected and lysed by phage Φ A72. Likewise, *Staph. aureus* Sa9 lysA72 cells containing Φ A72 as a prophage were immune to Φ A72 but susceptible to Φ H5 infection. Based on these results, Φ A72 and Φ H5 belong to a distinct immunity group and, thus, suitable mixtures of these phages would prevent the development of lysogenic derivative strains. Alternatively, another feasible strategy would be to obtain lytic derivative phages unable to lysogenize (García et al., 2007).

Antimicrobial Activity of Phages in Milk

Preliminary challenge trials were performed in UHT whole-fat milk to assess the ability of the phages to lyse *Staph. aureus* Sa9 individually or in combination. The growth of *Staph. aureus* was inhibited regardless

of whether single phages or a combination were used (Figure 2). However, the mixture was significantly more efficient ($P < 0.05$), most likely by preventing the selection of bacteriophage-insensitive mutants and lysogenic derivatives.

Staphylococcus aureus Sa9 was also challenged with the phage mixture in pasteurized, semi-skimmed, and whole-fat raw milks to determine to what extent commonly used milk treatment procedures (i.e., heating and skimming) affected the ability of the phages to inhibit *Staph. aureus* (Figure 3). In pasteurized whole-fat milk, the *Staph. aureus* population was 3.6 log units lower than the control culture without phages at the end of the incubation period. Accordingly, the phage titer increased with time (Figure 3A). An earlier effect of the phage mixture on *Staph. aureus* growth was observed in semi-skimmed raw milk because lower staphylococcal counts were detected compared with the control culture after 4 h of incubation. Differences were significant ($P < 0.05$) throughout the incubation period (Figure 3B). In contrast, the antibacterial activity of the phage mixture was clearly reduced in whole-fat raw milk (Figure 3C). Nevertheless, significantly lower counts were observed ($P < 0.05$) after 11 h. It is worth noting that in all cases, the phage mixture kept *Staph. aureus* counts low enough to avoid toxin accumulation over concentrations that cause food intoxication (Waldvogel, 2001), even in raw milk. This would be of great relevance to the food industry, as it would prevent accumulation of toxins that are not easily inactivated by heat treatments.

In all tested conditions, total clearance of *Staph. aureus* in milk was not achieved. This might be expected because of the very low phage/cell ratio used in the experiments or because of resistance development. It is widely recognized that at low cell densities, larger numbers of phages are required to ensure efficient infection (Kasman et al., 2002). The antibacterial activity of

Figure 2. Inhibitory activity of single phages and the phage mixture (10^4 to 10^5 pfu/mL) against *Staphylococcus aureus* Sa9 at 37°C in UHT whole-fat milk: ◇ = *Staph. aureus*, ■ = *Staph. aureus* + ΦA72, ▲ = *Staph. aureus* + ΦH5, ● = *Staph. aureus* + ΦA72 + ΦH5.

the phages could also be reduced by the slow growth of *Staph. aureus* in both raw milks, likely because of the antagonistic and competitive effect of milk microbiota. Furthermore, the complexity linked to untreated raw milks may hinder phage diffusion. This will clearly hinder the chance of a phage encountering its host.

We observed that phage titer decreased about 1 log unit throughout the incubation period in semi-skimmed and whole-fat raw milk assays (Figure 3B and Figure 3C). This could indicate partial inactivation of the phages and their progeny in unheated milks. The raw milk used in these assays was not homogenized, in con-

trast to the commercial UHT and pasteurized milks. Thus, fat globules and milk protein might aggregate phage particles and render them unable to infect the host under the assay conditions. Similar inactivation in raw milk has been described for bacteriophage K (O'Flaherty et al., 2005a; Gill et al., 2006). A more detailed analysis should be carried out to determine whether milk components protect cells from infection or the phages themselves are inactivated.

Antimicrobial Activity of Phages in Milk Under Storage and Breakdown Temperature

Because development of *Staph. aureus* is usually a consequence of a breakdown in the cold chain during milk processing, we also tested how phages influence the growth of the pathogen under refrigeration conditions followed by a shift to a warmer incubation. Refrigerated milk was contaminated with *Staph. aureus* Sa9 (10^2 cfu/mL) and a phage mixture (ΦH5 + ΦA72) at 10^1 , 10^2 , and 10^4 pfu/mL. Incubation was performed at 4°C for 18 h and the temperature was then increased to 22°C for the following 30 h. During cold storage of milk, nonappreciable changes in the viable counts and phage titer occurred (Figure 4). The shift to a permissive temperature resulted in exponential development of *Staph. aureus* in both control and phage-infected milk cultures. Nevertheless, lower pathogen growth clearly dependent on the phage concentration was observed in phage-infected cultures for the first 24 h of incubation at 22°C. At the end of the incubation period, however, a 5-log-unit reduction in bacterial counts compared with the control was observed regardless of the initial phage concentration (Figure 4A). The phage titer was stable at refrigeration temperatures and increased sharply after 12 h of incubation at 22°C. As previously reported

Figure 3. Antibacterial activity of phages ΦH5 and ΦA72 against *Staphylococcus aureus* Sa9 at 37°C in A) pasteurized whole-fat milk, B) semi-skimmed raw milk, and C) whole-fat raw milk. □ = *Staph. aureus*, ■ = *Staph. aureus* + ΦA72 + ΦH5, × = phage titer.

Figure 4. Inhibitory activity of a mixture of phages $\Phi H5$ and $\Phi A72$ against *Staphylococcus aureus* Sa9 in UHT whole-fat milk during a simulated breakdown in the cold chain. Incubation proceeded at 4°C for 18 h, shifted to 22°C, and was maintained there for 30 h. A) Growth of *Staph. aureus* Sa9 (◆ = *Staph. aureus*, ■ = *Staph. aureus* + phages $\Phi H5$ and $\Phi A72$ (10¹ pfu/mL), ▲ = *Staph. aureus* + phages $\Phi H5$ and $\Phi A72$ (10² pfu/mL), □ = *Staph. aureus* + phages $\Phi H5$ and $\Phi A72$ (10⁴ pfu/mL); B) titer of bacteriophages in samples in which phages were added at initial concentrations of 10¹ pfu/mL (■), 10² pfu/mL (▲), or 10⁴ pfu/mL (□).

(Cairns et al., 2009), phage propagation only occurred when *Staph. aureus* could multiply actively and reach a threshold bacterial concentration that allowed the phage titer to increase (Figure 4B). These results showed that bacteriophages might be used as an additional countermeasure against temperature breakdown.

CONCLUSIONS

Our findings suggest that the dairy environment is an extensive source of staphylococcal phages, which may be effective in certain milk types to inhibit staphylococcal strains that often cause mastitis and contaminate milk. However, all the isolated phages were temperate, a feature that may hinder their efficacy as biocontrol agents in food. The use of phage mixtures is advisable to cover a wider inhibitory spectrum and to avoid, at least to some extent, the development of resistance. Phages of dairy origin were shown to be suitable to counteract the harmful effects of temperature failure during milk processing and storage.

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