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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 ΦH5, ΦG7, and ΦA72, were found in 89% of the samples; all the isolated phages were temperate. Phages ΦH5 and ΦA72 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 ΦH5 and ΦA72

Staph. aureus strain	RAPD profile[1]	EOP	
		ΦH5	ΦA72
Sa1	A	0.72 ± 0.03	1.01 ± 0.07
Sa2	A	0	0.39 ± 0.02
Sa3	B	0	1.02 ± 0.18
Sa4	A	0	1.32 ± 0.06
Sa5	C	0	0
Sa8	C	0	0
Sa9	D	1 ± 0.05	1 ± 0.1
Sa10	E	0	0
Sa11	E	0	0.26 ± 0.05
Sa12	E	0	0
Sa13	E	0	0
Sa14	E	0	0
Sa15	E	0	0
Sa16	E	0	0

[1]Strains 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 Analysis 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 μL) 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 $CaCl_2$ and 10 mg/L $MgSO_4$. The cultures were incubated overnight at 37°C with shaking. Samples were centrifuged at 13,000 × 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 $MgSO_4$, 10 mg/L $CaCl_2$, 100 mg/L NaCl, pH 7.5) containing 50% glycerol (vol/vol). Phages were purified by ultracentrifugation (100,000 × 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
ΦC1	+	Cabrales cheese	3	<u>Sa1</u>	GDC6
ΦP1	+	Peñamellera cheese	1	<u>Sa9</u>	—
ΦL7	+	Milk	1	<u>Sa3</u>	AC9, FG1, AC11, GDC6, GRA16
ΦL13	+	Milk	1	Sa1, Sa2, <u>Sa4</u>, Sa9	AC9, DC6
ΦA8	+	Milk	2	Sa1, <u>Sa2</u>, Sa9, Sa11	—
ΦH5	+	Milk	26	<u>Sa1</u>, Sa9	AC9, FG1, AC11, GDC6, GRA16, JFL2,
ΦG7	+	Milk	20	Sa1, Sa9, <u>Sa11</u>	—
ΦA72	+	Milk	21	Sa1, Sa2, Sa3, Sa4, <u>Sa9</u>, Sa11	AC9, AFG1, AC11, GDC3, GDC6, GRA16, JFL2, JFL6

[1]Number 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.

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