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Phage sensitivity and prophage carriage in *Staphylococcus aureus* isolated from foods in Spain and New Zealand

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ABSTRACT

Bacteriophages (phages) are a promising tool for the biocontrol of pathogenic bacteria, including those contaminating food products and causing infectious diseases. However, the success of phage preparations is limited by the host ranges of their constituent phages. The phage resistance/sensitivity profile of eighty seven *Staphylococcus aureus* strains isolated in Spain and New Zealand from dairy, meat and seafood sources was determined for six phages (Φ11, K, ΦH5, ΦA72, CAPSa1 and CAPSa3). Most of the *S. aureus* strains were sensitive to phage K (*Myoviridae*) and CAPSa1 (*Siphoviridae*) regardless of their origin. There was a higher sensitivity of New Zealand *S. aureus* strains to phages isolated from both Spain (ΦH5 and ΦA72) and New Zealand (CAPSa1 and CAPSa3). Spanish phages had a higher infectivity on *S. aureus* strains of Spanish dairy origin, while Spanish strains isolated from other environments were more sensitive to New Zealand phages. Lysogeny was more prevalent in Spanish *S. aureus* compared to New Zealand strains. A multiplex PCR reaction, which detected ΦH5 and ΦA72 sequences, indicated a high prevalence of these prophages in Spanish *S. aureus* strains, but were infrequently detected in New Zealand strains. Overall, the correlation between phage resistance and lysogeny in *S. aureus* strains was found to be weak.

1. Introduction

Staphylococcus aureus is a serious threat to human health, due to its ability to cause a multitude of skin and respiratory infections and foodborne illnesses. It is part of the normal microbiota on human skin and in mucous, and is the main cause of *Staphylococcus* infections in hospitals (Figueiredo and Ferreira, 2014) and food contamination during handling (Wattinger et al., 2012). Its ability to form biofilms can lead to persistent contamination of food processing (Gutiérrez et al., 2012; Herrera et al., 2007; Spanu et al., 2013) and hospital environments (Otto, 2013). Recently, the exponential increase in livestock-associated methicillin-resistant *S. aureus* strains (LA-MRSA), such as clone CC398, have become a concern due to their emergence along the whole farm to fork chain (farm animals, meat product and humans) (Fluit, 2012). Moreover, the emergence of vancomycin resistant *S. aureus* strains (VRSA) narrows the antibiotic arsenal available to treat staphylococcal infections (Weigel et al., 2003).

Phages and phage lytic proteins have been proposed as alternative treatments to reduce food contamination and combat infections caused by pathogenic bacteria (García et al., 2010). Some phage-based products are already available in the market to be used in the food industry. These include Listex™ P100 (www.micreosfoodsafety.com) and ListShield™ (www.intralytix.com) that have been recognized as safe by the US Food and Drug Administration (FDA) and approved by the US Department of Agriculture (USDA) as antimicrobial processing aids to combat *Listeria monocytogenes* in foods, and on food processing surfaces.

One of the key factors for the success of phage-based products is likely to be a sufficiently wide host range to ensure efficacy against the majority, if not all, strains of the pathogen. Due to their specificity for certain receptors on the cell wall, phages typically have relatively a narrow host range, and so to overcome this, the use of phage mixtures is usually preferred (Chan et al., 2013; Hagens and Loessner, 2010). Another factor to be considered in the use of phage biocontrol in foods is lysogeny in the target bacterium. Prophages typically impart immunity to super-infection of related phages to the host cell (Berngruber et al., 2010), and so this could be a potential barrier to the successful use of phage biocontrol. Prophages are very often present in the chromosome of pathogenic bacteria, and the majority of *S. aureus* isolates harbor at least one prophage (Goerke et al., 2009).

Previously, we have characterized the temperate phages ΦA72 and ΦH5 isolated from the dairy environment in Spain, and their lytic

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derivatives, vB_SauS-phiIPLA35 and vB_SauS-phiIPLA88, both belonging to the *Siphoviridae* family (García et al., 2007, 2009a). These phages were able to inhibit *S. aureus* growth in milk, curd and cheese manufacturing processes (Bueno et al., 2012; García et al., 2007, 2009b). More recently, biocontrol candidate phages CAPSa1 and CAPSa3 were isolated from milk samples in New Zealand. They are virulent phages that belong to the *Siphoviridae* family (unpublished).

The present work aims to address the efficacy of biocontrol using phages and hosts from distant geographical areas such as Spain and New Zealand. To do this, we have determined phage sensitivity/resistance profiles in a representative *S. aureus* collection containing strains from both countries. In addition, we have characterized the incidence of lysogeny and the carriage of prophages ΦA72 and ΦH5 and its relationship with bacterial resistance.

2. Material and methods

2.1 Bacterial strains, phages, media and growth conditions

Sixty four *S. aureus* strains from three food environments (dairy, meat and seafood) were isolated in Spain, and 23 strains from dairy and one strain from meat were obtained from New Zealand (Table 1). Staphylococcal cells were isolated on Baird Parker Agar (BP) supplemented with egg yolk, and routinely cultured in TSB broth (Triptone Soy Broth, Scharlau) at 37 °C with shaking or in TSB plates containing 2% (w/v) bacteriological agar (TSA).

Phages were routinely propagated as previously described (García et al., 2007). Phage K (O'Flaherty et al., 2005), Φ11 (Iandolo et al., 2002), ΦA72 and ΦH5 were propagated in *S. aureus* Sa9, while *S. aureus* NZRM2016 was used as host strain for phages CAPSa1 and CAPSa3. Phage enumeration was performed by the double-layer technique (Gutiérrez et al., 2010) using soft TSA medium (0.7% agar plus 10 mM $CaCl_2$ and 10 mM $MgSO_4$) in the upper layer.

2.2 Lysogeny determination

The presence of resident prophages in the *S. aureus* strain collection was determined by mitomycin C induction as previously described (Gutiérrez et al., 2010). Briefly, mid-exponential-phase cultures were treated with 0.5 μg/ml of mitomycin C (Sigma-Aldrich, St. Louis, MO) for three hours at 37 °C and shaking. Supernatants were filtered and spotted into agar overlayed lawns of all the *S. aureus* strains.

2.3 Phage host range

The host range of each phage was obtained against a collection of *S. aureus* strains by spotting 5 μl (10^9 pfu/ml) of the phage suspension into the lawn of each strain using the double-layer technique. Efficiency of plating (EOP) was calculated using *S. aureus* Sa9 as the reference strain (Gutiérrez et al., 2010).

2.4 Multiplex PCR

The genomic nucleotide sequences of ΦA72 and ΦH5 (García et al., 2009a) were subjected to progressive MAUVE alignment, using the default settings (http://gel.ahabs.wisc.edu/mauve/). Regions with no homology were analyzed to design specific primers for each phage, and these primers were submitted to *in silico* PCR amplification (http://insilico.ehu.es/PCR/) to verify their specificity. For ΦA72, one pair of primers was designed surrounding the *orf2* (522 bp, from nucleotide 1350 to 1872) and another pair in the region corresponding to a methyl transferase, *orf22* (324 bp, from 11,331 to 11,655). For ΦH5, pairs of oligonucleotides were designed in *orf29* (704 bp, from 151 to 855) and in the integrase region, *orf1* (225 bp, from 13,013 to 13,238). Total DNA was extracted by GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich, Madrid, Spain), according to the manufacturer's instructions. PCR reactions were performed with PureTaq Ready-To-Go™ PCR Beads (GE Healthcare, Munich, Germany), 10 ng of DNA and 1 μM of each primer. PCR reactions were based on phage ΦA72 (GenBank NC_011612) using four primers (gp2F: 5′GATAATTACAACTGGGATACC3′; gp2R: 5′GTATTCAGACAATGTTTTGAAG3′; metrF 5′ATAGAATGCAACATTCACC3′; metrR 5′GATAACAACCATTCTGGTAC3′) and the other based on ΦH5 (GenBank NC_011614) (int88F:5′ ATCATTGTGTAATAGATAAGAGC3′; int88R: 5′GTTATTACAGATAAAGCTTATGC3′; gp29F: 5′CATGATTGAAGAGACCATC3′; gp29R: 5′CTACTGCGTCATTTAAATTTC3′). As positive control, pure phage DNA from ΦA72 and ΦH5 was used. PCR was performed in a thermocycler (Bio-Rad, Hercules, USA) under the following thermal cycling conditions: one cycle at 95 °C for 5 min; 35 cycles at 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min; and a final step of 10 min at 75 °C.

2.5 Statistical analyses

Statistical analyses were performed using R (R Core Team, 2013).The unpaired *t*-test and Chi-square test was conducted to compare the sensitivity/resistance of staphylococcal strains isolated in Spain and New Zealand to six phages. The Chi-square test was used to compare the sensitivity/resistance of Spanish staphylococcal strains isolated from different food environments (dairy, meat, seafood) to the same phages. A significance level of 0.05 was chosen for these purposes. Fisher's exact two tailed test was run using R (R Core Team, 2013), where the null hypothesis was that phage sensitivity and lysogeny are independent. McNemar's Chi-squared test with continuity correction was also run on R (R Core Team, 2013), with the null hypothesis being the proportion

Table 1
Strains used in this study, geographical isolation and origin.

Country	Food Industry	Origin	*S. aureus* strain	Reference
Spain	Dairy 1	Milk	Sa1, Sa2, Sa3, Sa4, Sa5, Sa6, Sa8, Sa9, Sa10, Sa11, Sa12, Sa13, Sa14, Sa15, Sa16	García et al., 2009b
	Dairy 2	Milk	AAAC9, AAAC10, AAAC11, AFG1, AFG2, GDC3, GDC6, GDC9, GRA16, GRA17, GRA20, JFL2, JFL4, JFL6, JFL8	
	Dairy 3	Milk	IPLA19, IPLA20, IPLA24	Unpublished
	Dairy 4	Food-contact surfaces	IPLA1, IPLA3	Gutiérrez et al., 2012
	Meat 1	Food-contact surfaces	IPLA5, IPLA6, IPLA7, IPLA8, IPLA13, IPLA14, IPLA15, IPLA16, IPLA17, IPLA18	
	Seafood	Food-contact surfaces	IIM201, IIM208, IIM214, IIM222, IIM228, IIM229, IIM233, IIM234, IIM235, IIM237, IIM238, IIM239, IIM240, IIM241, IIM242, IIM245, IIM246 IIM249	
New Zealand	Dairy 5	Raw milk	PHCFAP1, PHCFAP2, PHCFAP3, S34, S36, S38, S39, S41, S43, S45, S46, S47	Unpublished
	Dairy 6	Cheese	S52, S51, FM31, FM34	
	Dairy 7	Milk powder	S100, S12	
		Skimmed milk powder	S27, S28	
		Dairy product	S14	
		Cream pie	NZRM3372	
	Meat 2	Ham	NZRM3374A	
	Bovine	–	NZRM2016	