Molecular Characterisation of Bacteriophage K Towards Applications for the Biocontrol of Pathogenic Staphylococci

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Summary

The aim of this work was to characterise staphylococcal bacteriophage (a bacterial virus) and to assess their potential as therapeutic agents against pathogenic strains of *Staphylococcus aureus,* particularly mastitis-causing strains. The project included the use of two newly isolated phage CS1 and DW2, and an existing polyvalent phage. The new phage were isolated from the farmyard and characterised by electron microscopy and restriction analysis. Both phage were shown to belong to the *Siphoviridae* family and were lytic for representatives of all three clonal groups of Irish mastitis-associated staphylococci. A cocktail of three phage (CS1, DW2 and K) at 10^8 (plaque forming units) PFU/ml was infused into cows teats in animal trials. The lack of an increase in somatic cell counts in milks indicated strongly that the phage did not irritate the animal. In addition, the most potent phage used in this study, phage K, was further studied by genome sequencing, which revealed a linear DNA genome of 127,395 base pairs, which encodes 118 putative ORFs (open reading frames). Interesting features of the genome include; 1) a region exhibiting high homology to the structural module from *Listeria* phage A511, 2) genes which potentially encodes proteins necessary for its own replisome, 3) an absence of GATC sites and 4) three introns encoding putative endonucleases were located in the genome, (two in the putative DNA polymerase gene and one in the lysin gene). Unlike both CS1 and DW2, the polyvalent phage K, exhibited a broad host range within the genus *Staphylococcus.* In *in vitro* inhibitory assays, phage K lysed all staphylococcal strains tested including nine different species. In preliminary application-type studies, anti-staphylococcal activity was also evident in a hand wash

and phage cream. An unexpected result was the observation that phage K was unable to replicate in raw milk, which could limit its applications in mastitis treatments. This may have been due to clumping of the bacteria caused by immunoglobulins. However, inhibition activity was lost after milk was heat-treated. The overall results in this study provide new insights into the biology of the broad host range phage K and indicate that phage K has potential for treatment and prevention of infections caused by pathogenic staphylococci.

Introduction

The prevalence of antibiotic resistance in the pathogen *Staphylococcus aureus* is a major problem, given that the bacterium causes a wide variety of human and animal infections. Infections in humans caused by *S. aureus* include primary infections such as osteomyelitis, impetigo, septicaemia, and secondary infections such as eczema and decubitus ulcers (Noble 1998). Mastitis in dairy cattle is the most common cause of death in adult dairy cows (Bradley 2002) causing estimated losses worldwide of 35 billion US dollars annually (Wellenberg *et al.* 2002). Staphylococcal mastitis, in particular, can be manifested by clinical and subclinical infection that can persist throughout the lactation period and into subsequent lactations (Kerro Dego *et al.* 2002). Such infections are generally not treated satisfactorily by antibiotic therapy in eliminating existing disease or in preventing the establishment of chronic infection (Sol *et al.* 2000; Kerro Dego *et al.* 2002). The pathogenic potential of *Staphylococcus* is greatly aided by its ability to develop resistance to antibiotics (Lowy 2003). Indeed, more than 90% of strains are now believed to be penicillin resistant (Rubin *et al.* 1999) and consequently methicillin has become one of the drugs of choice for treating infections. However, the emergence of methicillin resistant *Staphylococcus aureus* (MRSA) (Lowy 2003) has led to the use of vancomycin to treat these MRSA strains. Therefore, the more recent reports of vancomycin resistant *S. aureus* (VRSA) strains are of obvious concern (Lowy 2003).

Investigations for new and alternative anti-microbials effective against *S. aureus* have become increasingly relevant for both human and veterinary applications. Bacteriophage (phage) are obligate intracellular parasites that multiply inside bacteria and in so doing make use of the host biosynthetic machinery. Phage come in many shapes and sizes and have either DNA or RNA as their genetic material. This DNA or RNA genome is encapsulated in a protein coat, which is normally connected to a

base-plate by a tail. Phage were investigated to eliminate bacteria as far back as 1921, including staphylococci in human infections (Chanishvili *et al.* 2001). The aim of this project was to characterise staphylococcal bacteriophage and to evaluate their potential as therapeutic agents against pathogenic strains of *Staphylococcus aureus*.

1. Isolation and characterisation of two anti-staphylococcal bacteriophages specific for pathogenic *Staphylococcus aureus* associated with bovine infections

Summary

We describe the isolation of two anti-staphylococcal phage (viruses which infect *S. aureus*) namely DW2 and CS1 from farmyard slurry using the strain DPC5246. Both phage were characterised by electron microscopy and restriction analysis and shown to belong to the *Siphoviridae* family. CS1 and DW2 were lytic for representatives of all three clonal groups of Irish mastitis-associated staphylococci. Infection of *S. aureus* DPC5246 (10^6 (colony forming units) CFU ml^{-1}) with CS1 and DW2 alone or in combination resulted in kill rates of 99.5%. A cocktail of three phage (CS1, DW2 and K) at 10^8 PFU /ml was infused into cow's teats in animal trials. The observation that there was no detectable increase in somatic cell counts (SCCs) in milks indicates that the phage did not irritate the mammary tissue.

Results

Selection of *Staphylococcus aureus* host strains for phage isolation

S. aureus isolates from bovine mastitis infections in Ireland were previously divided into three major clonal/RAPD (Randomly amplified polymorphic DNA) groups, namely RAPD group 5, 7 and 4 (Fitzgerald *et al.* 1997), with a representative strain for each group classified as DPC5245, DPC5246 and DPC5247, respectively (Twomey *et al.* 2000). *S. aureus* DPC5246 was used for the initial screening of environmental samples for phage. It was later found that phage which were identified using this strain could also form plaques on strains DPC5245 and DPC5247.

Isolation of wild-type phage from farmyard slurry

Twenty farmyard slurry samples from a variety of locations in different farms were screened for the presence of phage, which were capable of forming plaques on

strain *S. aureus* DPC5246. Five samples gave positive results, in that non-turbid plaques were visible in lawns of strain DPC5246. These plaques were isolated and propagated on strain DPC5246 allowing the extraction of phage DNA from each phage for restriction analysis. Restriction endonucleases, which gave clear digests included *Xba*I and *Xho*I, (Fig. 1b). Comparison of restriction digests indicated that there were only two distinct phage as evidenced by the *Xba*I digests shown in Figure 1. These were designated DW2 and CS1. Three of the phage gave identical patterns to either DW2 or CS1 and thus these three were excluded from the study. The previously characterised phage K was included in the restriction analysis for comparison. Different restriction patterns were observed for each phage, CS1 and DW2, when compared to phage K (Fig. 1b, lane 2, 4 and 6), whereas, restriction patterns of phage CS1 and DW2 had some similarities indicating that both phage are related at the genetic level (Fig. 1b).

Characterization of phage CS1 and DW2

The plaque-forming ability of phage CS1 and DW2 were compared. Phage CS1 and DW2 formed clear plaques of 1 mm in diameter on strain DPC5246. Morphological analysis of phage DW2 and CS1 by electron microscopy (Fig. 1) allowed each to be classified into its respective viral family and order. Both phage have an obvious tail and hence belong to the order *Caudovirales.* Phage CS1 has a non-contractile flexible tail 112.5 +/- 2.5 nm in length and an isometric head 40.8 +/- 3.8 nm in diameter, a base plate extending from the end of the tail can also be observed (Fig. 1, i). DW2 has a similar morphology to CS1 with a long flexible tail 120 +/- 10 nm in length and an isometric head 39.8 +/- 0.76 nm in diameter (Fig. 1, ii). Both CS1 and DW2 can therefore be classified into the *Siphoviridae* family based on guidelines of the International Committee on Taxonomy of Viruses (Murphy 1995).

Bacteriophage host range

The potential efficacy of CS1 and DW2 to be used in combination with phage K was studied in a cocktail for control of bovine mastitis staphylococci. The host range of CS1 and DW2 were therefore assessed and compared to that of phage K. Both CS1 and DW2 were found to lyse the representatives of the three groups of bovine *S. aureus* isolates. In addition, a number of human *S. aureus* isolates were

also included to help establish the extent of the host range (Table 1). These strains are MRSA isolates, which were previously sourced from human infections in Irish hospitals and previously shown to be distinct from each other (Cotter *et al.* 1998). Details of the bacterial strains are shown in Table 1. The host range of phage CS1 and DW2 while not identical are similar with both phage capable of lysing some of the human strains. In contrast, phage K lysed all strains tested. Phage DW2 lysed the three bovine clonal types and their plaquing ability on these strains was quantified in terms of EOP (efficiency of plaquing) values. The results demonstrate that DW2 plaqued on DPC5246 (the propagating strain) with an EOP of 1.0, on DPC5245 with an EOP of 1.3×10^{-4} and on DPC5247 with and EOP of 7.6×10^{-2}. It is expected that if DPC5245 or DPC5247 were used as the propagating strain, the EOP data would vary accordingly (Coffey and Ross 2002). This phage was only weakly lytic against the human strains in that it did not form discernable plaques on these human strains although there was inhibition of bacterial growth on the lower dilutions of the plaque assay and also lysis by the spot test technique. In the case of phage CS1, it plaqued on DPC5246 (the propagating strain) with an EOP of 1.0, on strain DPC5245 with an EOP of 6.0×10^{-3} and on DPC5247 with an EOP of 2.7×10^{-1}. This phage, like phage DW2 is also weakly lytic against the human-derived strains. The EOP data above suggests that strain DPC5245 possesses restriction modification activity (Coffey and Ross 2002) against CS1 and DW2 phage.

Bacterial challenge tests

Both phage CS1 and DW2 were assessed for their ability to lyse the mastitis causing-staphylococcal strain DPC5246 alone and in combination. Lysis of strain DPC5246 was monitored by plate count in triplicate. Each phage and a combination of CS1 and DW2 was added at a MOI of 10 to a mid-exponential culture of *S. aureus* DPC5246 at 37ºC. At this point the culture was at a concentration of approximately 10^6 CFU ml^{-1}. Three hours after phage addition, regardless of whether a single phage or both in combination was added, the bacterial numbers were reduced 10,000-fold (4 logs) when compared to the control where no phage had been added (Fig. 2). Thus, the combination of CS1 and DW2 did not increase the killing effect over and above either when used singularly (Fig. 2).

Potential of phage to irritate bovine mammary tissue

A 10 mM sodium phosphate medium (pH 7) was employed as a carrier medium for infusion of phage in the bovine mammary gland. When 100 µl of the carrier medium without phage was infused into the teat sinus, no increase in the somatic cell count (SCC) was observed in milks obtained in subsequent milkings indicating no immune response. Following this, the carrier medium was used to deliver a cocktail of the three phage (CS1, DW2 and K, titer 10^8 PFU /ml) together also in a volume of 100 µl into the teat sinus. This also resulted in no significant increase in the SCC. SCCs of 1.83×10^5 were recorded in the test teat (right front) whereas, the SCC in the non-infused 3 control teats were 1.93×10^5, 1.81×10^5 and 7.9×10^4, respectively in the four milkings taken during the two days post-infusion. This clearly indicates that high-titre phage preparations do not induce a local immune response and are therefore non-irritating to the animal.

Discussion and Conclusions

This study identified two new phage against bovine staphylococci, which were isolated from farmyard slurry against the bovine strain DPC5246, which is a representative of one of the three major clonal types that cause *S. aureus* mastitis in Ireland. Phage CS1 and DW2 were assigned to the *Siphoviridae* family based on morphology. Both phage were capable of forming plaques on the bovine strains, whereas, no discernable plaques were found against the human strains. The two phage CS1 and DW2 were each capable of reducing staphylococcal counts 10,000 fold at an MOI (multiplicity of infection) of 10. The inclusion of the polyvalent phage K enhanced the anti-bacterial effect. We showed that infusion of the phage cocktail into the bovine udder did not cause the SCC to rise above 400,000 /ml, indicating that there was no local immune response to high numbers of phage. In conclusion, we have identified two new phage against bovine staphylococci, which may be combined with anti-staphylococcal phage K. This study confirms that the application of phage to the udder at high titer does not pose an irritation problem for the animal. It thus opens the way for the incorporation of phage into teat-dips or teat-washes as a prophylactic against bovine staphylococci which may naturally reside on teat surfaces and thus may have the potential to cause mastitis.

2. Inhibition of phage proliferation in raw bovine milk

Summary

The ability of bacteriophage (phage) to replicate in milk is important in situations where phage might be used as a therapeutic for bovine mastitis. Phage K was able to replicate normally, leading to the elimination of the host culture in milk, which had been previously heat-treated. When raw milk was used under identical conditions, the phage were unable to replicate. Phage adsorption assays were performed and these demonstrated that adsorption of phage was significantly reduced in the raw milk while it was restored in the heat-treated sample (86.5% compared to 99.96% adsorption, respectively). When confocal microscopy with a LIVE/DEAD *Bac*light staining system was employed, it was observed that in raw milk *S. aureus* formed clusters associated with fat globules, while in heat-treated milk, bacterial agglutination had not occurred.

Results and Discussion

Staphylococcal phage inhibition in raw milk and dry cow secretion

The lytic activity of phage K was examined in detail using *S. aureus* DPC5645. Growth of this bacterium was therefore measured by plate count in BHI broth, HT milk, raw milk and dry cow secretion. Phage was added 4 h after bacterial inoculation. In the case of BHI and HT milk, the bacterial numbers declined to undetectable levels within 2 h. In the case of the raw milk and the dry-cow secretion, there was no detectable reduction in bacterial numbers (Fig. 3).

To explain the inhibition of the phage activity in the raw milk and dry-cow secretion, bacterial adsorption tests were performed and the results compared for raw milk and HT milk. Essentially, free phage numbers were assessed 15 min after addition of phage to milks containing *S. aureus* DPC5246. It was observed that there were far higher numbers of free phage present in the raw milk supernatant compared to the HT milk supernatant indicating that the phage adsorption process was being inhibited in the raw milk. Indeed, 99.96% of phage K was adsorbed by *S. aureus* DPC5246 in HT milk compared to 86.5% of phage in raw milk, which represents a 1,000 fold decrease of adsorption in raw milk. These results suggest that some heat-sensitive component in the raw milk is preventing the adsorption of phage to cells and

it also indicates that the phage particles themselves are not being inactivated given that they are still capable of plaquing during the plaque assay stage of the adsorption test. The phage inhibitor is thus likely to be associated with the bacterial cells themselves.

In situ staining of *S. aureus* DPC5246

In situ LIVE/DEAD *Bac*Light stain which shows red fluorescence for dead bacteria and green fluorescence for living bacteria was employed with Confocal Scanning Laser Microscopy (CSLM) for analysing the behaviour of *S. aureus* DPC5246 in raw and HT milk before and 60 min after addition of staphylococcal phage K. In the case of the raw milk, staphylococcal cells were organised in clumps and all cells were alive (Fig. 4a). After addition of phage K, the majority of staphylococci in the clump were still alive, although one or two dead cells are evident within some of the clumps (Fig. 4b). In the case of the HT milk, staphylococcal cell clumping was not evident as the cells were distributed throughout the sample (Fig 4c). One hour after addition of phage K, it was difficult to locate cells within the sample and any intact bacterial cells that were visualised were stained red and were thus dead (Fig. 4d). These results correlate with the cell counts during phage challenge in the different milks. Cell clumping is known to be due to the action of immunoglobulins of the M class or agglutinins in the raw milk and this activity is heat sensitive (Walstra and Jenness 1984) and also that some bacterial species are more sensitive to this immunoglobulin activity than others. It would, appear therefore that the phage resistance is not necessarily due to the clumping *per se*, but alternatively could be connected to immunoglobulins coating the bacterial cell surface and preventing adsorption of bacteriophage.

Phage challenges in homogenised milk and whey

Examination of *S. aureus* DPC5246 by CSLM, clearly indicated cell agglutination (Fig. 4a). Many of these clumps appeared to be associated with fat globules and it has also been reported that immunoglobulins bind bacteria to fat globules (Walstra and Jenness 1984). Challenges were performed in homogenised milk to investigate if the fat globules in raw milk were responsible for the reduced phage activity. Raw milk was homogenised and a phage challenge was performed in the homogenised sample. During the homogenisation protocol milk was heated to

45°C prior to homogenisation; hence, a control challenge was also performed in milk heated to 45°C. The inability of phage K to inhibit DPC5645 at 45°C indicated that the protein inhibiting phage adsorption was still functional after heat treatment at this temperature. It was found that homogenisation of the milk did not greatly improve the phage activity against staphylococci in the raw milk. Assays were also performed in whey, the aqueous fraction of milk remaining after the removal of fat and casein. As with the above assays, phage proliferation in heated and unheated whey was tested. In the HT whey phage K reduced bacterial numbers to undetectable levels within 2 h. In contrast, when the whey was unheated, phage K activity was inhibited, but not completely inhibited as seen in the raw milk. In this case, the phage did reduce the bacterial numbers to undetectable levels within 4 hours. Therefore inhibition of phage activity is still present in the whey, but it appears that the removal of the fat and various other milk proteins during whey production has reduced the effect.

Conclusions

Bacteriophage K is a candidate phage for therapeutic applications both for humans and animals. The phage has previously been included in washes to reduce numbers of staphylococci (O'Flaherty *et al.* 2005). In addition, we explored its infusion into bovine teats for mastitis control. In this context, the efficacy of the phage was examined in dry-cow secretions and also in raw milk. It was consistently observed that the phage killing effect disappeared in both of these media. In conclusion, investigation of phage K activity in raw bovine milk shows that the host bacterium for the phage is organised into clumps due to agglutinins active in the raw milk. The ability of phage K to lyse these bacteria is inhibited, unless the milk is heated. It is likely therefore, that heat-labile immunoglobulins, which recognise and bind to the bacterial cell surface, are responsible for this effect. Thus, the use of phage to eliminate these pathogens, which are recognised by these milk immunoglobulins, would most likely be impeded in natural milk and udder secretions.

3. Potential of the polyvalent anti-*Staphylococcus* bacteriophage K for the control of antibiotic-resistant staphylococci.

Summary

In this study, phage K was assessed *in vitro* for its ability to inhibit emerging drug resistant *Staphylococcus aureus* strains from hospitals and other species of *Staphylococcus* isolated from bovine infections. In *in vitro* inhibitory assays, phage K lysed a range of methicillin-resistant *S. aureus* (MRSA) strains, *S. aureus* with heterogeneous-vancomycin and vancomycin resistance as well as teicoplanin resistant strains. In these assays, 14 of the MRSA strains were initially only weakly sensitive to this phage. However, propagation of phage K on these less-sensitive strains resulted in all 14 being sensitive to the modified phage. Model *in situ* hand-wash studies using a phage-enriched wash solution resulted in a 100-fold reduction in staphylococcal numbers on human skin by comparison with numbers remaining after washing in phage-free solution. Infusion of the phage into a non-immunogenic bismuth-based cream resulted in strong anti-*Staphylococcus* activity from the cream on agar plates and broth assays.

Results

Phage K inhibits recently emerged drug resistant bacteria

To test the host range and potency of phage K (Fig. 5) bacterial challenge experiments were performed. Details of the bacterial strains are shown in Table 2. These include an *S. aureus* type strain, 36 human MRSA strains, 4 glycopeptide resistant strains, 4 distinct clinical isolates from bovine mastitis and 8 coagulase-negative non-*S. aureus* species of *Staphylococcus*. The MRSA strains have previously been shown by motif-dependant PCR to be distinct (M. Daly, personal communication; (Cotter *et al.* 1998). Of the 53 strains, 39 were successfully lysed by phage K as indicated by phage spot test and confirmed by plaque assay (Table 2). 14 of the strains from the MRSA group were relatively insensitive to phage K in the initial challenge (Table 2). Plaque formation did not occur with any of these when phage K was used, although there was inhibition in the lawn of bacterial growth, typically at phage concentrations of 108, 107 and 106 PFU/ml by using the plaque assay technique. This inhibition of growth in the lower dilutions occurred with all the apparently insensitive MRSA strains. When phage K was incubated with these strains

in broth, modified phage K variants, which were capable of forming clear plaques on their respective hosts could be obtained for all of the 14 insensitive strains (Table 2).

In addition, two of the modified phage, namely phage K.W64352 and phage K.W65216 were assayed for their ability to lyse or cross-react with all the other phage insensitive strains in the study. Where phage K.W64352 was used, normal plaque formation was evident on the majority of strains, but notably, plaques were pinpoint on strains W65216, M249180, 254959 and M251955, but the plaque numbers (EOP) were similar to those obtained on strain DPC5246. These pinpoint plaques suggest that these strains may have phage resistance systems in addition to restriction modification (r/m), which only permit a relatively low burst size. Similarly, when phage K.W65216 was used, plaques were faint and pinpoint on strains W64352 (propagating host for phage K.W64352), M249138, M255409 and W69939. As with phage K.W64352, efficiency of plaquing on these strains was similar to that on strain DPC5246. This indicates that r/m is the principal cause of the phage insensitivity in the 14 isolates. It also indicates that there is common specificity among the r/m systems harboured by these strains.

Bacterial challenge experiments

Challenge experiments were performed in BHI broth with MRSA strain DPC5645. This involved adding phage K to an exponential culture of DPC5645 at 37°C. The potency of phage K is illustrated in this challenge experiment, within 30 min phage K started to reduce bacterial numbers and within 2 h the phage had reduced the MRSA isolate (DPC5645) from 5.7 x 10^6 CFU/ml to an undetectable level. Plate counts confirmed that there were no viable bacteria remaining in the BHI 10 h after phage treatment indicating that no BIMs had formed during the challenge. Indeed, further plate counts 25 h after phage infection confirmed the absence of BIMs in this experiment. The absence of BIMs from this experiment agreed with the results of plaque assay procedure to detect and enumerate BIMs, in that at a MOI of 1, BIM formation did not occur on plates.

Inhibition of *S. aureus* on skin by phage wash.

Phage K was assessed for its ability to reduce the numbers of MRSA on human skin using *S. aureus* DPC5246 as a test strain. These trials demonstrated that

washing in phage-free ringers solution was associated with a slight reduction in the number of challenge organisms. When phage K was included in the wash at a titre of 1.4 x 10^8 PFU/ml, the number of staphylococci remaining was reduced a further 100-fold (Fig. 5).

Phage inhibition of *S. aureus* in a bismuth-based cream.

Phage K also exhibited inhibition of the indicator strain DPC5246 in a bismuth-based cream. The phage cream was placed in the centre of an overlaid plate with the indicator strain DPC5246. A zone of inhibition is seen surrounding the phage cream (Fig. 7A), which shows that the cream containing phage has killed the surrounding bacteria by bacterolysis. Importantly, the phage cream was also shown to kill the indicator strain when placed in broth cultures of the challenge organism. Following a 4 h incubation at 37°C, the control sample contained 8 x 10^7 CFU/ml of DPC5246 (turbid Fig. 7B, i) whereas no viable bacteria were detected in the test sample (clear Fig. 7B, ii), showing complete kill by the phage cream within 4 h.

Discussion and Conclusions

In this study we demonstrate that phage K inhibits 9 different species of *Staphylococcus*, namely, *S. aureus*, *S. epidermidis*, *S. saprophyticus*, *S. chromogenes*, *S. captis*, *S. hominis*, *S. haemolyticus*, *S. caprea* and *S. hyicus*. Within *S. aureus*, it is inhibitory to a wide range of distinct strains from different hospital and veterinary sources which were isolated over the last 3 years. We feel that these strains are representative of the problematic strains presently associated with infections in Ireland. Of particular interest is the inhibitory effect on recently emerged methicillin-resistant strains (obtained from hospital staff, out-patients and in-patients). These studies show that phage K could be modified to inhibit less-sensitive strains, especially MRSA, with better efficiency strains simply by passing the phage through the target strain, which ordinarily would not allow plaque formation. These data suggests the presence of restriction modification activity in these MRSA isolates and also that there is a large degree of common specificity among the r/m systems harboured by these strains. The modified phage generated in this study could be combined with phage K in a cocktail to increase the host-range of the phage preparation. The bacterial challenge using phage K indicated that no bacteria

remained 2 h after phage addition in cases where up to 10^7 CFU/ml were used. It is apparent from the results that washing hands in the presence of phage K has the potential to significantly reduce the numbers of problematic *S. aureus* strains that are resident on human hands. With regard to the inclusion of phage in the cream, its anti-bacterial effect is self-evident from the *in-vitro* experiment described. This suggests that such phage creams could find applications in treatment of local skin infections.

In this study we demonstrate that the exclusively lytic phage K (25) has particular applications in the prevention and/or treatment of infections caused by antibiotic-resistant staphylococci. In this respect, we have shown its ability to kill a broad range of newly isolated pathogenic staphylococci including both human and veterinary strains. Moreover, the study details some preliminary findings, which show the potential of delivering the phage in an anti-staphylococcal cream or hand wash.

4. Genome of staphylococcal phage K: a new lineage of *Myoviridae* infecting low G+C content Gram positive bacteria.

Summary

Phage K is a polyvalent phage of the *Myoviridae* family which is active against a wide range of staphylococci. Phage genome sequencing revealed a linear DNA genome of 127,395 base pairs, which encodes 118 putative open reading frames. The genome is organised in a modular form, encoding modules for lysis, structural proteins, DNA replication and transcription. Interestingly, the structural module shows high homology to the structural module from *Listeria* phage A511, suggestive of intergenus horizontal transfer. In addition, phage K exhibits the potential to encode proteins necessary for its own replisome, including DNA ligase, primase, helicase, polymerase, RNase H and DNA binding proteins. Phage K has a complete absence of GATC sites making it insensitive to restriction enzymes which cleave this sequence. Three introns (*lys*-I1, *pol*-I2 and *pol*-I3) encoding putative endonucleases were located in the genome. Two of these (*pol*-I2 and *pol*-I3) were found to interrupt the DNA polymerase gene while another (*lys*-I1) interrupts the lysin gene. Two of the introns encode putative proteins with homology to HNH endonucleases, whereas the other encodes a 270 aa protein, which contains two zinc fingers ($CX_2CX_{22}CX_2C$ and $CX_2CX_{23}CX_2C$). The availability of the genome of this

highly virulent phage, which is active against infective staphylococci, should provide new insights into the biology and evolution of large broad-spectrum polyvalent phage.

Results and Discussion

General features of the genome of phage K

The phage K genome is presented as a 127,395 bp contiguous sequence of linear double-stranded DNA which encodes at least 118 putative ORFs, which were capable of encoding peptides of at least 100 amino acids in all six reading frames preceded by a potential Shine-Dalgarno sequence at a distance of at least 3-18 bp from a start codon (AUG, GUG or UUG) (Table 3). The majority of ORFs (112) initiate translation with the AUG start codon, whereas only 5 (ORFs 38, 40, 41, 42 and 96) initiate translation with the UUG start codon and 1 (ORF 63) initiates with a GUG start codon (Table 3). Bioinformatic analysis of ORFs revealed that the majority exhibited low identities with proteins from the database (Table 3), which often is the case with new genomes. The genome can be divided into two distinct regions, which are divergently transcribed as indicated by bioinformatic analysis. In this respect, of the 118 ORFs, 85 are transcribed in one orientation with 33 in the opposite, with all of the latter grouped together in the first 30 kb as illustrated in Fig. 8. Phage K has a G + C content of 30.6% which is significantly lower than that generally associated with the staphylococcal bacterial genome.

Phage K has its genes arranged in modules

Temperate staphylococcal phage are generally organised in a modular form which include modules for lysogeny, DNA replication, transcriptional regulation, packaging, structural proteins and lysis. The organisation of these temperate staphylococcal phage genomes is similar to those of temperate streptococcal phage. Phage K also appears to have its genes arranged in modules but the order differs from the temperate phage and the two sequenced lytic phage which have their lysis module embedded in the structural region. The modules of phage K are not as well defined as those of the temperate staphylococcal and streptococcal phage; for example, there is a lack of intergenic regions between the structural and DNA replication and transcription modules. Putative rho-independent terminators were identified (Table 2) using the TransTerm program (Ermolaeva *et al.* 2000). Three further terminators were located upstream of ORFs 1, 56 and 118 on the divergent strand to the ORFs.

These terminators are characterised by a stem loop in the mRNA followed by a U-rich sequence and allow for a punctuation of the 3' ends of multi-cistronic mRNA.

Taxonomy and comparative genomics

The need for a genome based taxomony tree has recently been identified (Rohwer and Edwards 2002). After studying 3,981 proteins of 105 genomes no single gene that could be used as a basis for a classification system was found in all phages. Instead a taxonomic system was based on a predicted phage proteome. The current phage proteome taxonomy is based on both complete phage genomes and prophage identified from within bacterial genomes (Casjens 2003). The database consists of 16,260 proteins from 375 genomes. The ICTV family Myoviridae grouped together in their system with the exception of T4 and P4 coliphage. These two phage represent their own groups in the proteomic tree indicating that they are the only sequenced representatives. Likewise, phage K does not fall within a defined group, confirming that it is the founding member of a new taxonomic group, and that the Myoviridae are more diverse than their visual characterisations suggest.

Lysis module is located in the first divergently transcribed 30 kb

The lysis module (ORFs 30 to 33) is located at the end of the first 30 kb where all ORFs are divergently transcribed in relation to the rest of the genome (Fig. 8). ORF 33 encodes a putative holin of 167 aa (18.1 kDa) whose stop codon overlaps by 1 bp in a different reading frame ORF 32. Both ORFs 33 and 32 have recognisable ribosome binding sites (Table 3). The putative holin of phage K exhibited 61% identity with a holin from phage Twort (Table 3) and probably functions by generating pores in the bacterial cell membrane. The lysin (spliced products of ORFs 30 and 32, see below) contains the recently described CHAP domain which is characterised by three conserved motifs (Bateman and Rawlings 2003; Rigden *et al.* 2003).

Introns with ORFs interrupting genes with crucial enzymatic functions

Analysis of the genome revealed that both the putative polymerase and lysin genetic determinants contained intron-like sequences. Indeed, the polymerase gene contained two such putative structures (*pol*-I2 and *pol*-I3), each encoding endonucleases (ORF 87 [I-*Ksa*II] and ORF 89 [I-*Ksa*III], respectively) (Fig. 9A). In

contrast, the lysin gene contained one intron like sequence (*lys*-I1), which also encodes a distinct endonuclease (ORF 31 [I-*Ksa*I]) (Fig. 9B). Both I-*Ksa*I and I-*Ksa*III exhibit homology to HNH endonucleases (Table 1) and contain a HNH motif. Interestingly I-*Ksa*I also contains an intron-encoded nuclease repeat motif at the C-terminal end (data not shown). The functions of the nuclease repeats are unknown but could be involved in DNA binding via the helix-turn-helix motif (residues 116 to 164). I-*Ksa*II exhibited no significant homology to any protein in the database. Closer examination of I-*Ksa*II revealed the existence of two potential zinc-binding motifs ($CX_2CX_{23}CX_2C$ and $CX_2C_{22}CX_2C$) and thus it may belong to a subfamily of HNH endonucleases containing a zinc-binding motif.

Overview of relationship to other phage: phage K and *Listeria* phage A511 have similar structural modules.

When structural proteins of phage K were examined by SDS-PAGE (Fig. 10), four were identified that correspond to predicted proteins of phage K (ORF 44, 49, 50 and 95), on the basis of N-terminal sequencing. N-terminal sequencing identified the putative major tail sheath protein ORF 49 of phage K which is 54.5 kDa (Fig. 10). The amino acid sequence matched the first 7 N-terminal amino acids of ORF 49 except for the initial methionine. Database searches revealed a 57% identity with the tail sheath protein of *Listeria* phage A511 (Fig. 10), which is a member of the *Myoviridae* family, has a contractile tail, linear double-stranded DNA, and a large genome of 116 kb (Loessner and Scherer 1995). Only its structural module and amidase have been sequenced to date, and it cannot, therefore, be included on the proteome tree. N-terminal sequencing of band B resulted in 15 amino acids which are identical to residues 25 to 39 of the deduced protein product of ORF 44, indicating postranslational cleavage of the first 23 amino acids. This ORF shares 82% identity with the capsid protein of phage Twort (Table 3). ORF 44 shares 66% identity with the capsid protein of phage A511 which also exhibits postranslational cleavage of the first 23 amino acids (Loessner and Scherer 1995) (Fig. 10). Interestingly band C corresponds to a protein of unknown function (ORF 95) (Fig. 10). N-terminal sequencing revealed the first 17 amino acids of this protein, which has a predicted molecular mass of 23.2 kDa (Fig. 10). The amino acid sequence obtained from band D (Fig. 10) corresponds to ORF 50 with a predicted molecular mass of 15.9 kDa.

ORF 50 shares an identity of 68 % with ORF 8 from *Listeria* phage A511 which has an unknown function (Loessner and Scherer 1995).

The proteins within the structural module are not homologous to the equivalent proteins of the sequenced lytic and temperate staphylococcal phage but they do exhibit homology to *Listera* phage A511, with the exception of ORF 41. Interestingly this 11,361 bp (minus the portal protein) structural region of phage K shows significant homology with phage A511 not just at gene level but also in the arrangement of ORFs (Loessner and Scherer 1995) (Fig. 11). The dramatic similarity between this large (11 kb) region of both phage would suggest that phage K and A511 are related and could constitute a new lineage of *Myoviridae* infecting low-G+C gram-positive bacteria. The elucidation of the genome of A5ll and other large *Myoviridae* may facilitate the classification of these large *Myoviridae* to the same lineage.

Conclusions

Phage K is a large, virulent bacteriophage, which infects a broad range of staphylococci including multiple drug resistant strains of *S. aureus*. Detailed genetic characterisation of this phage has unveiled a number of features as follows. (i) Phage K has been taxonomically placed in its own group because of overall uniqueness when compared to other phage. (ii) The genome also contains introns in essential phage functions, two in the polymerase and one in the lysin genes. (iii) Phage K contains a large region with remarkable homology to *Listeria* phage A511. (iv) Finally, phage K has a remarkable paucity of GATC and GGNCC, sites suggesting phage K has evolved an efficient counter defense against host restriction-modification systems.

Overall Conclusions

The resurgence in the study of bacteriophage as potential therapeutics is clearly evident given both the increased number of publications on the subject and the commercial interest devoted to them, including the setting up of a numbebr of small companies. In this respect, phage may prove to have an important role to play in killing pathogens such as *S. aureus*, a major cause of infections in humans and animals. In this study, three different phage were investigated for their potential as anti-bacterial agents against *S. aureus*. Two of these, CS1 and DW2 were isolated as

part of this study and the third, phage K was purchased from a culture collection. Phage K was the most potent of the three phage since it could kill MRSA strains, coagulase negative staphylococci and mastitic causing *S. aureus* strains compared to CS1 and DW2, which were specific for *S. aureus* bovine mastitis-causing strains. No local immune response was observed in the bovine udder, suggesting that the phage cocktail could be safely applied and tolerated *via* this route. Subsequently, the ability of phage K to replicate in raw milk was studied with a view to developing anti-mastitis applications. Surprisingly, we found that raw milk inhibited phage K proliferation, while heat-treated milk supported phage K infection and multiplication. This inhibition is possibly due to the clumping state of the cells caused by immunoglobulins. We feel that this observation has important ramifications for mastitis applications of phage therapy.

While phage therapy shows tremendous potential for treatment of staphylococcal infections, it is fair to say that there is an urgent need for scientifically conducted double blind placebo-controlled trials to prove efficacy. Although phage may not replace antibiotics in clinical practice, they may find their own niche in the treatment of certain infections, which are difficult to treat with antibiotics. Furthermore, the use of new technologies and molecular techniques allows the screening of phage genomes for additional anti-bacterials and new drug discovery targets (Liu *et al.* 2004). In this respect it is interesting that the genome of phage K encodes up to 200 genes of which approximately only 30% are homologous to protein sequences in the database. Compare this situation to bacterial genomics where it is common to find that greater than 70% of the predicted proteins have known homologues. This alone demonstrates how much we have to learn about the biology and function of these fascinating intracellular parasites.

Bibliography

Bateman, A. and Rawlings, N.D. (2003) The CHAP domain: a large family of amidases including GSP amidase and peptidoglycan hydrolases. *Trends Biochem Sci* **28**, 234-237.

Bradley, A. (2002) Bovine mastitis: an evolving disease. *Vet J* **164**, 116-128.

Casjens, S. (2003) Prophages and bacterial genomics: what have we learned so far? *Mol Microbiol* **49**, 277-300.

Chanishvili, N., Chanishvili, T., Tediashvili, M. and Barrow, P.A. (2001) Phages and their appplication against drug-resistant bacteria. *J Chem Technol Biotechnol* **76**, 689- 699.

Coffey, A. and Ross, R.P. (2002) Bacteriophage-resistance systems in dairy starter strains: molecular analysis to application. *Antonie Van Leeuwenhoek* **82**, 303-321.

Cotter, L., Daly, M., Greer, P., Cryan, B. and Fanning, S. (1998) Motif-dependent DNA analysis of a methicillin-resistant *Staphylococcus aureus* collection. *Br J Biomed Sci* **55**, 99-106.

Ermolaeva, M.D., Khalak, H.G., White, O., Smith, H.O. and Salzberg, S.L. (2000) Prediction of transcription terminators in bacterial genomes. *J Mol Biol* **301**, 27-33.

Fitzgerald, J.R., Meaney, W.J., Hartigan, P.J., Smyth, C.J. and Kapur, V. (1997) Fine-structure molecular epidemiological analysis of *Staphylococcus aureus* recovered from cows. *Epidemiol Infect* **119**, 261-269.

Kerro Dego, O., van Dijk, J.E. and Nederbragt, H. (2002) Factors involved in the early pathogenesis of bovine *Staphylococcus aureus* mastitis with emphasis on bacterial adhesion and invasion. A review. *Vet Q* **24**, 181-198.

Liu, J., Dehbi, M., Moeck, G., Arhin, F., Bauda, P., Bergeron, D., Callejo, M., Ferretti, V., Ha, N., Kwan, T., McCarty, J., Srikumar, R., Williams, D., Wu, J.J., Gros, P., Pelletier, J. and DuBow, M. (2004) Antimicrobial drug discovery through bacteriophage genomics. *Nat. Biotechnol.* **22**, 185-191.

Loessner, M.J.and Scherer, S. (1995) Organization and transcriptional analysis of the Listeria phage A511 late gene region comprising the major capsid and tail sheath protein genes cps and tsh. *J Bacteriol* **177**, 6601-6609.

Lowy, F.D. (2003) Antimicrobial resistance: the example of *Staphylococcus aureus*. *J Clin Invest* **111**, 1265-1273.

Murphy, F.A., Fauquet, C.M., Bishop, D.H.L., Ghabrial, S.A., Jarvis, A.W., Martelli, G.P., Mayo, M.A., Summers, M.D. (1995) Virus Taxonomy. In *Virus Taxonomy: Sixth Report of the International Committee on Taxonomy of Viruses*. (ed). Wien: Springer-Verlag.

Noble, W.C. (1998) Staphylococcal Diseases. In *Topley and Wilsons Microbiology and Microbial Infections*. Vol. 3. Collier, L., Balows, A. and Sussman, M. (eds), pp. 231-256.

O'Flaherty, S., Ross, R.P., Meaney, W., Fitzgerald, G.F., Elbreki, M.F. and Coffey, A. (2005) Potential of the polyvalent anti-*Staphylococcus* bacteriophage K for the control of antibiotic-resistant staphylococci from hospitals. *Appl Environ Microbiol* **71:**1836-1842.

Rigden, D.J., Jedrzejas, M.J. and Galperin, M.Y. (2003) Amidase domains from bacterial and phage autolysins define a family of gamma-D,L-glutamate-specific amidohydrolases. *Trends Biochem Sci* **28**, 230-234.

Rohwer, F.and Edwards, R. (2002) The Phage Proteomic Tree: a genome-based taxonomy for phage. *J Bacteriol* **184**, 4529-4535.

Rubin, R.J., Harrington, C.A., Poon, A., Dietrich, K., Greene, J.A. and Moiduddin, A. (1999) The economic impact of *Staphylococcus aureus* infection in New York City hospitals. *Emerg Infect Dis* **5**, 9-17.

Sol, J., Sampimon, O.C., Barkema, H.W. and Schukken, Y.H. (2000) Factors associated with cure after therapy of clinical mastitis caused by *Staphylococcus aureus*. *J Dairy Sci* **83**, 278-284.

Twomey, D.P., Wheelock, A.I., Flynn, J., Meaney, W.J., Hill, C. and Ross, R.P. (2000) Protection against *Staphylococcus aureus* mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin, lacticin 3147. *J Dairy Sci* **83**, 1981-1988.

Walstra, P.and Jenness, R. (1984) *Dairy Chemistry and Physics*: John Wiley and Sons.

Wellenberg, G.J., van der Poel, W.H. and Van Oirschot, J.T. (2002) Viral infections and bovine mastitis: a review. *Vet Microbiol* **88**, 27-45.

Publications arising from this project

Peer reviewed papers

- S. O'Flaherty, A. Coffey, R. Edwards, W. Meaney, G.F. Fitzgerald and R.P. Ross. (2004). Genome of Staphylococcal Phage K: a New Lineage of *Myoviridae* Infecting Gram-Positive Bacteria with a Low G+C Content. **Journal of Bacteriology** 186:2862-2871.

- S. O'Flaherty, R.P Ross, W. Meaney, G.F. Fitzgerald, M. F. Elbreki and A. Coffey. (2005). Potential of the polyvalent anti-*Staphylococcus* bacteriophage K for the control of antibiotic-resistant staphylococci from hospitals. **Applied and Environmental Microbiology** 71(4) 1836-1842

- S. O'Flaherty, R.P. Ross, J. Flynn, W.J. Meaney, G.F. Fitzgerald and A. Coffey. (2005). Isolation and characterization of two anti-*staphylococcal* bacteriophage specific for pathogenic *Staphylococcus aureus* associated with bovine infections. **Letters in Applied Microbiology** 41(3), 482-486

- S. O'Flaherty, A. Coffey, W. Meaney, G.F. Fitzgerald and R.P. Ross. (2005). Inhibition of phage proliferation in raw bovine milk. **Letters in Applied Microbiology** 41(3), 274–279

- S. O'Flaherty, A. Coffey, W. Meaney, G.F. Fitzgerald and R.P. Ross. (2005). The recombinant phage lysin LysK has a broad spectrum of lytic activity against clinically relevant staphylococci including MRSA **Journal of Bacteriology** 187: 7161-716

Abstracts

- S. O'Flaherty, Coffey, A., Meaney W., Fitzgerald G.F. and Ross R.P. (2005). Heterologous expression of *lysK* encoding a lysin with anti-MRSA activity

from the genome of the anti-staphylococcal phage K. 15th European Congress of Clinical Microbiology and Infectious Diseases.

- S. O'Flaherty, Ross, R. P., Meaney, W. J., Fitzgerald, G. F., Elbreki, M., & Coffey, A. (2004). Evaluation of bacteriophage and bacteriophage lysin for the elimination of drug-resistant clinically-relevant *Staphylococcus aureus*. Procedings of the 2nd International Symposium on Resistant Gram-positive Infections. Berlin, Germany, December 2004 p 29.

- S. O'Flaherty, Coffey, A., Meaney W., Fitzgerald G.F. and Ross R.P. (2004). Cloning and heterologous expression of a cDNA encoding LysK; the lysin identified in the genome of the polyvalent anti-staphylococcal bacteriophage K. ASM Conference on the New Phage Biology, Florida, USA.

- S. O'Flaherty, A. Coffey, W. Meaney, G.F. Fitzgerald M. F. Elbreki and R.P Ross. (2004). Bacteriophage phage K inhibits a broad range of drug-resistant clinically relevant staphylococci and has potential for incorporation into hand wash and skin cream preparations. ASM Conference on the New Phage Biology, Florida, USA.

- S. O'Flaherty, Ross, R. P., Elbrecki, M., Meaney, W. J., Fitzgerald, G. F., & Coffey, A. (2004). Exploitation of polyvalent bacteriophage K for the control of drug-resistant clinically-relevant Staphylococci. Society For General Microbiology Annual Meeting, Trinity College Dublin, September 2004.

- S. O'Flaherty, Coffey, A., Meaney W., Fitzgerald G.F. and Ross R.P. (2004) The war against *Staphylococcus*-could phage be the new WMD (Weapons of Microbial Destruction)? Walsh Fellowship Seminar. RDS Dublin 11th November.

- Coffey, O'Flaherty, S., Meaney, W., Elbrecki, M., & Ross, R.P. (2004) Use of polyvalent anti-staphylococcal bacteriophages for the biocontrol of

methicillin-resistant *Staphylococcus aureus* and other staphylococci. Clinical Microbiology and Infection 10 (Suppl.3): 135-136.

- S. O'Flaherty, Meaney, W. J., Fitzgerald, G.F. Ross, R.P., and Coffey, A (2003). The genome sequence of the polyvalent anti-*Staphylococcus* bacteriophage K provides insights into phage biology and intron invasion. *Irish Journal of Agricultural and Food Research* (Abstract).

- S. O'Flaherty, Meaney, W. J., Fitzgerald, G.F. Coffey, A. and Ross, R.P. (2003). The polyvalent anti-*Staphylococcus* bacteriophage K exhibits activity against a broad range of clinically relevant Staphylococci including MRSA. *Irish Journal of Agricultural and Food Research* (Abstract).

- S. O'Flaherty, W. J. Meaney, R. P. Ross, J. Flynn, G.F. Fitzgerald and A.Coffey (2003). Evaluation of anti-staphylococccal bacteriophages as alternatives to antibiotics in the control of bovine mastitis. *Agricultural Research Forum*, *Tullamore, 3 & 4 March 2003*, page 21.

- Coffey, O' Flaherty, S., Meaney, W., Elbrecki, M., & Ross, R.P. (2003) Biocontrol of Methicillin Resistant *Staphylococcus aureus* (MRSA) using polyvalent bacteriophages. Proceedings of BioNet Annual Conference 2003. Pages 22-23.

- S. O'Flaherty, Meaney, W. J., Ross, R.P., Flynn, J. Fitzgerald, G.F. and Coffey, A (2002). Isolation and characterisation of anti-staphylococcal bacteriophages for the control of bovine mastitis. *Irish Journal of Agricultural and Food Research* 41: 130 (Abstract)

Presentations

- The war against *Staphylococcus*-could phage be the new WMD (Weapons of Microbial Destruction)? Walsh Fellowship Seminar. RDS Dublin 11th November 2004. S. O'Flaherty.

- Cloning and heterologous expression of a cDNA encoding LysK; the lysin identified in the genome of the polyvalent anti-staphylococcal bacteriophage K. Presented at the ASM conference on the New Phage Biology, Florida, USA, August 2004. (S. O'Flaherty)

- Evaluation of anti-staphylococccal bacteriophages as alternatives to antibiotics in the control of bovine mastitis. Presented at the Agricultural Research Forum, Tullamore, 3 & 4 March 2003. (S. O'Flaherty)

- Isolation and characterisation of anti-staphylococcal bacteriophages for the control of bovine mastitis. Presented at the 31nd Food Science and Technology Research Conference, UCC, September 2001. (S. O'Flaherty)

Theses

- **PhD Thesis:** Sarah O'Flaherty (2005). Molecular Characterisation of Bacteriophage K and the Lysin LysK Towards Applications for the Biocontrol of Pathogenic Staphylococci., NUI University College Cork

Other

- Travel award to attend the ASM conference on New Phage Biology in Florida, USA, August, 2004. (S. O'Flaherty).

- Cover photograph of the Journal of Bacteriology 2004, 186:2862-2871.

Table 1: Host range of phages CS1 and DW2 compared to phage K

S. aureus strain	Strain details	Phage CS1	Phage DW2	Phage K
DPC5245	Bovine RAPD 5, DPC Culture Collection	+	+	+
DPC5246	Bovine RAPD 7, DPC Culture Collection	+	+	+
DPC5247	Bovine RAPD 4, DPC Culture Collection	+	+	+
W64352	MRSA, CIT culture collection (2001)	-	-	+
M249318	MRSA, CIT culture collection (2001)	+	+	+
M255039	MRSA, Waterford Regional Hospital (2001)	-	+	+
MS 811	MRSA, Cork University Hospital (2003)	+	-	+
89715	MRSA, Cork University Hospital (1999)	+	+	+

+ susceptible to phage by spot test
- not susceptible to phage by spot test
MRSA: Methicillin Resistant *S. aureus*
CIT: Cork Institute of Technology
DPC: Dairy Products Research Centre
RAPD: Randomly arbitrarily primed DNA

Table 2: Phage K sensitivity and details of bacterial strains

Host	Strain	Strain Details[i]	Methicillin Sensitivity[h]	Phage sensitivity[f]	EOP[g]	Phage sensitivity after modification	EOP after phage modification
S. aureus	8325	Type strain[a]	S	+	nc		
S. aureus	St3550	Teicoplanin resistant[a]	S	+	0.087		
S. aureus	St2573	Teicoplanin resistant[a]	R	+	0.11		
S. aureus	Mu50	VRSA[a]	R	+	nc		
S. aureus	Mu3	hVRSA[a]	R	+	nc		
S. aureus	M249318	Human MRSA[b]	R	-		+	6.75×10^{-5}
S. aureus	W64352	Human MRSA[b]	R	-		+	2.3×10^{-1}
S. aureus	W65216	Human MRSA[b]	R	-		+	2.8×10^{-4}
S. aureus	M231003	Human MRSA[b]	R	+	1.03×10^{-1}		
S. aureus	M249180	Human MRSA[b]	R	-		+	1.09×10^{-3}
S. aureus	MS811	Human MRSA[b]	R	-		+	2.26×10^{-3}
S. aureus	DPC5646	Human MRSA[b]	R	+	0.77		
S. aureus	DPC5645	Human MRSA[b]	R	+	0.45		
S. aureus	DPC5647	Human MRSA[b]	R	+	8.46×10^{-7}		
S. aureus	M249954	Human MRSA[c]	R	+	1.12×10^{-1}		
S. aureus	M250594	Human MRSA[c]	R	+	3.23×10^{-1}		
S. aureus	M254959	Human MRSA[c]	R	-		+	7.33×10^{-5}
S. aureus	M255039	Human MRSA[c]	R	-		+	1
S. aureus	M255409	Human MRSA[c]	R	-		+	7.6×10^{-2}
S. aureus	M253472	Human MRSA[c]	R	+	1		
S. aureus	M249739	Human MRSA[c]	R	+	1.57×10^{-1}		
S. aureus	M249892	Human MRSA[c]	R	+	4.10×10^{-1}		
S. aureus	M252776	Human MRSA[c]	R	+	1		
S. aureus	M251760	Human MRSA[c]	R	+	1.32×10^{-1}		
S. aureus	W71683	Human MRSA[c]	R	+	5.89×10^{-2}		
S. aureus	M253206	Human MRSA[c]	R	+	8.57×10^{-2}		
S. aureus	W73365	Human MRSA[c]	R	-		+	3.4×10^{-1}
S. aureus	M253470	Human MRSA[c]	R	+	6.93×10^{-1}		
S. aureus	M249025	Human MRSA[c]	R	+	1.41×10^{-1}		
S. aureus	M249138	Human MRSA[c]	R	+	1		
S. aureus	M249807	Human MRSA[c]	R	+	1.48×10^{-1}		
S. aureus	M250108	Human MRSA[c]	R	+	7.3×10^{-1}		
S. aureus	M249671	Human MRSA[c]	R	-		+	1.46×10^{-4}
S. aureus	W69939	Human MRSA[c]	R	-		+	1.177×10^{-3}
S. aureus	M253164	Human MRSA[c]	R	-		+	2.65×10^{-2}
S. aureus	M249678	Human MRSA[c]	R	-		+	1.75×10^{-4}
S. aureus	M251955	Human MRSA[c]	R	-		+	2.1×10^{-1}
S. aureus	M250564	Human MRSA[c]	R	+	5.1×10^{-3}		
S. aureus	MM77438	Human MRSA[c]	R	+	2.76×10^{-2}		
S. aureus	MM257671	Human MRSA[c]	R	+	5.46×10^{-3}		
S. aureus	MM234150	Human MRSA[c]	R	+	6.2×10^{-1}		
S. aureus	DPC5245	Bovine[d]	S	+	1		
S. aureus	DPC5246	Bovine[d]	S	+	1		
S. aureus	DPC5247	Bovine[d]	S	+	1		
S. aureus	DPC5971	Bovine[d]	S	+	0.21		
S. epidermidis	DPC6010[e]	Bovine[d]	S	+	0.46		
S. saprophyticus	DPC6011[e]	Bovine[d]	S	+	0.025		
S. chromogenes	DPC6012[e]	Bovine[d]	S	+	0.16		
S. captis	DPC6013[e]	Bovine[d]	S	+	nc		
S. hominis	DPC6014[e]	Bovine[d]	S	+	2.1×10^{-8}		
S. haemolyticus	DPC6015[e]	Bovine[d]	S	+	nc		
S. caprea	DPC6016[e]	Bovine[d]	S	+	0.022		
S. hyicus	DPC6017[e]	Bovine[d]	S	+	0.087		

a Public Health Laboratory Service. *b* Cork University Hospital, Cork, Ireland. *c* Waterford Regional Hospital, Waterford, Ireland. *d* Dairy Products Center, Fermoy, County Cork, Ireland.
e Coagulase negative. *f* Spot assay results. +, phage sensitive; -, not phage sensitive. *g* EOP, efficiency of plaquing; NC, not countable (plaques are too small to count, but confluent lysis occurs at $>10^7$ PFU/ml). *h* Sensitivity to methicillin at 5 µg/ml. S, sensitive; R, resistant. *i* VRSA, vancomycin-resistant *S. aureus;* hVRSA, heterogeneous vancomycin-resistant *S. aureus*.

Table 3. General features of putative ORFs from phage K with best matches in the database.

ORF	Start bp	Stop bp	Protein size kDa	Representative similarity to proteins in database	e Value	% identity (% match of length)	Accession no.
1*	2934	2449	18.4	Hypothetical protein			
2*	3358	2927	16.7	Hypothetical protein			
3	3914	3372	21.5	Hypothetical protein			
4*	4414		19.5	*Pseudomonas aeruginosa*, conserved hypothetical protein	3e-24	39 (40)	NP_253270
5*	4825	4427	16.1	Hypothetical protein			
6*	5529	4822	27.7	*Agrobacterium tumefaciens*, serine/threonine protein phosphatase I	2e-13	29 (26)	NP_534038
7*‡	6183	5629	21.2	Hypothetical protein			
8*‡	8050	7502	21.9	Hypothetical protein			
9*	9194	8457	28.7	Hypothetical protein			
10*	10003	9614	15.2	Hypothetical protein			
11*‡	10798	10316	18.8	Hypothetical protein			
12*‡	11390	10848	20.4	Hypothetical protein			
13*	11923	11390	20.7	Hypothetical protein			
14*	13213	12368	31.7	Hypothetical protein			
15*	13809	13225	21.9	*Mycoplasma penetrans*, AAA family ATPase	5e-20	40 (32)	NP_757966
16*	15233	14817	16	Hypothetical protein			
17*‡	15669	15367	11.3	*Enterococcus hirae*, ArpR	6e-18	45 (45)	CAA90708
18*	18110	16062	79.8	*Enterococcus faecalis*, plasmid pAD1, unknown protein	3e-04	28 (6)	AAL59463
19*‡	19226	18648	21.4	Hypothetical protein			
20*	19845	19219	23.8	Hypothetical protein			
21*	20734	19838	35	*Clostridium acetobutylicum*, homolog of eukaryotic DNA ligase III	3e-05	27 (14)	NP_347388
22*	21767	21027	28.6	*Neisseria meningitidis*, PhoH-related protein	1e-14	36 (38)	NP_273886
23*	22433	21819	23	*Staphylococcus aureus*, temperate phage phiSLT, orf636	0.51	26 (18)	NP_075517
24*	22874	22449	15.8	*Clostridium perfringens*, Ribonuclease H1	7e-14	34 (34)	NP_562366
25*	23719	23078	24.6	Hypothetical protein			
26*‡	24971	24279	24.8	*Staphylococcus aureus*, hypothetical protein	7e-07	29 (25)	NP_372619
27*	25793	25158	24.8	*Streptococcus pyogenes*, hypothetical phage protein	2e-22	36 (37)	NP_607354
28*‡	26651	25860	29.3	*Synechocystis sp*., unknown protein	1e-15	24 (20)	NP_440390
29*	26959	26651	12.2	Hypothetical protein			
30‡	27701	27072	23.1	*Staphylococcus aureus*, Autolysin (N-acetylmuramoyl-L-alanine amidase)	1e-54	52 (52)	P24556
31*	28472	27972	19.2	*Bacteroides thetaiotaomicron*, endonuclease	1e-12	39 (33)	NP_810939
32*	29435	28632	29.8	*Staphylococcus aureus*, phage Twort, N-acetylmuramoyl-L-alanine amidase	1e-38	40 (37)	CAA69021
33*	29938	29435	18.1	*Staphylococcus aureus*, phage Twort, holTW (holin)	9e-38	61 (53)	CAA69020
34*	34507	34833	12.3	Hypothetical protein			
35*	34848	36665	70.2	*Ralstonia solanacearum*, probale bacteriophage-related protein	0.002	25 (8)	NP_518974
36*	36658	37479	30.7	Hypothetical protein			
37*‡	37636	38115	18.5	Hypothetical protein			
38*†‡	38157	39350	43.6	*Bacillus cereus*, cell surface protein	3e-05	28 (24)	NP_346725
39*	39435	39776	12.8	Hypothetical protein			
40†	39794	40165	14.5	Hypothetical protein			
41*†‡	40169	41860	64.0	*Escherichia coli*, bacteriophage P27, putative portal protein	0.004	22 (10)	NP_543090
42*†	42054	42827	28.6	*Listeria monocytogenes*, bacteriophage A511, orf 1	2e-62	54 (63)	CAA62538
43*	42846	43796	35.7	Hypothetical protein			
44‡	43912	45303	51.2	*Staphylococcus aureus*, phage twort capsid protein	0.0	82 (81)	AAQ6728
				Listeria monocytogenes, bacteriophage A511, major capsid protein	e-174	66 (67)	CAA62540
45*	45704	46612	31.2	*Listeria monocytogenes*, bacteriophage A511, orf 3	e-104	60 (36)	CAA62541
46*	46626	47504	33.7	*Listeria monocytogenes*, bacteriophage A511, orf 4	8e-56	26 (9)	CAA62542
47*	47504	48124	23.8	*Listeria monocytogenes*, bacteriophage A511, orf 5	2e-25	46 (35)	CAA62543
48*	48143	48979	31.8	*Listeria monocytogenes*, bacteriophage A511, orf 6	6e-53	38 (39)	CAA62544
49*	49223	50986	54.5	*Listeria monocytogenes*, bacteriophage A511, major tail sheath protein	0.0	57 (58)	CAA62546
50*‡	51059	51487	15.9	*Staphylococcus aureus*, phage twort, unknown	3e-62	85 (84)	AF132670
				Listeria monocytogenes, bacteriophage A511, orf 8	5e-48	68 (67)	CAA62547
51*	51767	52225	18.1	Hypothetical protein			
52*	52514	52825	12.3	Hypothetical protein			
53*	52957	53415	18.1	*Listeria monocytogenes*, bacteriophage A511, orf 9	1e-23	39 (39)	CAA62548
54*‡	53459	53995	20.9	Hypothetical protein			
55	54051	58106	144	*Lactococcus lactis*, bacteiophage CP-1, orf 18	4e-08	32 (3)	NP_047298
56	58185	60611	91.2	*Staphylococcus epidermidis*, secretory antigen SsaA-like protein	4e-32	49 (13)	NP_765044
57*	60625	61512	34.6	Hypothetical protein			
58*	61512	64058	96.1	*Bacillus subtilis*, glycerophosphoryl diester phosphodiesterase	1e-41	38 (11)	NP_388095
59*	64165	64956	29.3	Hypothetical protein			
60*	64956	65480	20	Hypothetical protein			

61*	65480	66184	26.6	*Salmonella typhi*., putative bacteriophage baseplate protein	0.020	45 (7)	NP_456045
62	66199	67245	39.2	*Clostridium tetani*, phage-like element pbsx protein xkdT	5e-06	24 (43)	NP_782676
63†	67266	70325	116.2	Hypothetical protein			
64	70436	70957	19.2	Hypothetical protein			
65*‡	70978	74436	129	*Dichelobacter nodosus*, vrlC protein	9e-33	27 (10)	T17382
66*	74644	76566	72.5	*Staphylococcus aureus*, SLT orf 488-like protein	0.021	29 (4)	AAL82326
67*	76589	76963	14.6	Hypothetical protein			
68*‡	76970	78346	50.4	*Listeria monocytogenes*, lmo1188	0.034	24 (8)	NP_464713
69*	78438	80186	37.2	*Salmonella typhi*., putative helicase	2e-36	33 (16)	NP_569550
70*	80198	81811	63.2	*Enterococcus faecalis*, putative Rep protein	0.031	40 (5)	CAC29157
71*	81804	83246	54.6	*Salmonella typhi*., putative DNA helicase	4e-11	21 (15)	NP_569513
72	83325	84362	40.1	*Listeria monocytogenes*, similar to putative exonucleases SbcD	1e-09	23 (19)	NP_465171
73*	84362	84739	14.9	Hypothetical protein			
74*	84739	86658	73.4	*Yersinia pestis*, plasmid pMT1, probable exonuclease	2e-21	34 (9)	T14925
75*	86658	87254	23.2	Hypothetical protein			
76*	87269	88336	40.9	*Helicobacter pylori*, DNA primase	5e-07	24 (17)	NP_206814
77*	88741	89193	17	Hypothetical protein			
78	89180	89788	23.6	*Bacteriophage T5*, D14 protein	1e-05	26 (17)	O48499
79*	89805	90197	14.7	*Staphylococcus epidermidis*, NrdI protein	3e-13	35 (50)	NP_764067
80	90212	92326	80.2	*Staphylococcus aureus*, phage Twort, large ribonucleotide reductase subunit	0.0	72 (73)	AAM00816
81	92340	93389	40.4	*Staphylococcus aureus*, ribonucleoside reductase minor subunit	3e-84	53 (48)	NP_371256
82*	93407	93736	12.4	Hypothetical protein			
83	93720	94040	12	*Alicyclobacillus acidocaldarius*, Thioredoxin	0.013	33 (31)	P80579
84	94247	94843	23.5	Hypothetical protein			
85*‡	94853	95158	11.9	*Chlamydophila pneumoniae*, Integration Host Factor Alpha	0.003	26 (26)	NP_224616
86*	95234	96106	33.2	*Bacillus subtilis*, Bacteriophage SPO1, DNA polymerase	0.054	25 (12)	P30314
87*	96314	96784	18.6	Hypothetical protein			
88	96920	98263	52.8	*Thermotoga maritima*, DNA-directed DNA polymerase I	1e-06	22 (13)	NP_229419
89	98429	99238	31.2	*Bacteriophage Bastille*, HNH endonuclease, I-BasI	6e-22	40 (40)	AAO93095
90	99472	100332	32.9	*Bacillus subtilis*, phage SPO1, DNA -directed DNA polymerase	2e-31	36 (31)	JC1269
91*	100660	101142	18.9	Hypothetical protein			
92*‡	101229	102500	46.9	Hypothetical protein			
93*	102560	103816	46.8	*Thermotoga maritima*, DNA repair protein	2e-27	30 (24)	NP_229655
94*	104160	104822	26.6	*Bacillus subtilis*, Bacteriophage SPO1,RNA Pol sigma GP34 Factor	3e-05	32 (17)	P06227
95*	104950	105582	23.2	Hypothetical protein			
96*‡	105605	106117	17.8	*Staphylococcus aureus*, temperate phage phiSLT, major tail protein	4e-14	40 (49)	NP_075510
97*	106719	107474	29.2	Hypothetical protein			
98	107467	108717	47.5	Hypothetical protein			
99	108731	109099	14	Hypothetical protein			
100*‡	109086	109397	12	Hypothetical protein			
101*	109990	110757	30	*Staphylococcus aureus*, hypothetical protein	0.001	36 (16)	CAB60746
102*	110735	111181	17.3	Hypothetical protein			
103*	112416	113147	28.4	Hypothetical protein			
104*	113165	113623	17.9	Hypothetical protein			
105*	113688	114131	17.5	Hypothetical protein			
106*‡	114148	114852	27.4	Hypothetical protein			
107*‡	114914	115312	15.4	Hypothetical protein			
108*‡	115706	116176	17.9	Hypothetical protein			
109*	117041	117349	12	Hypothetical protein			
110*	117346	118254	35.2	*Pyrococcus horikoshii*, ribose-phosphate pyrophosphokinase	8e-09	27 (24)	NP_143754
111*‡	118272	119741	56.1	*Haemophilus ducreyi*, putative nicotinamide phosphoribosyl transferase	e-141	52 (52)	AAK06405
112*	120082	120477	15.5	Hypothetical protein			
113	121090	121401	11.7	Hypothetical protein			
114*	121407	121706	11.6	Hypothetical protein			
115*	122253	122606	13.9	Hypothetical protein			
116*	122625	123011	15.6	Hypothetical protein			
117*	124587	124913	12.7	Hypothetical protein			
118*	125811	126158	13.7	Hypothetical protein			

†, non AUG start codons
‡, presence of putative rho-independent terminators downstream of the ORF
*, good RBS sites upstream of the start codon

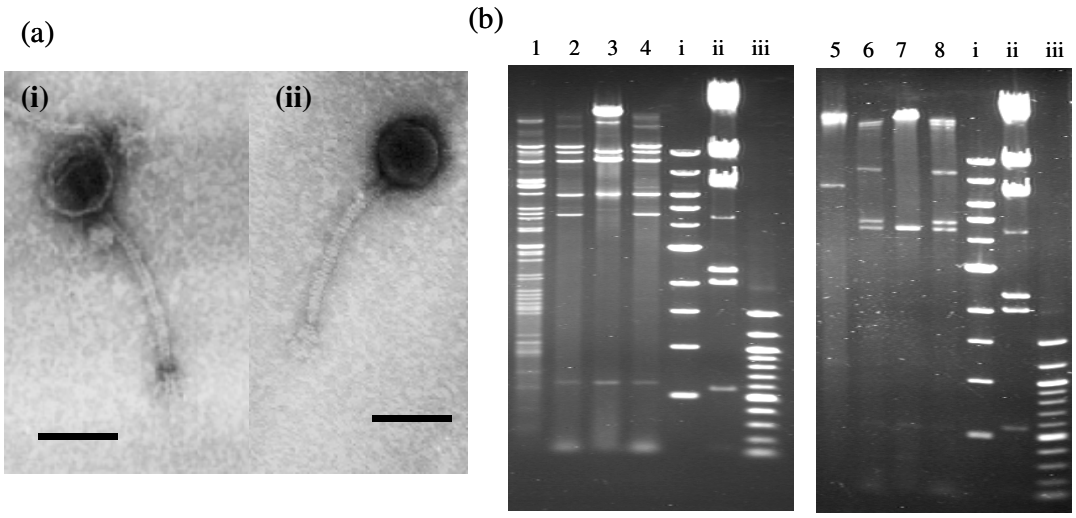


Figure 1

(a) Electron micrographs of phage CS1 and DW2. Phage were negatively stained with 1% uranyl acetate. (i) phage CS1, (ii) phage DW2. Scale bar represents 50 nm. (b) Phage DNAs digested with restriction endonucleases. Lane identification is as follows: 1, *Xba*1 digest of phage K; 2 and 4 *Xba*1 digest of CS1; 3, *Xba*1 digest of DW2, 5, *Xho*1 digest of phage K; 6 and 8 *Xho*1 digest of phage CS1 and 7, *Xho*1 digest of phage DW2. Lanes marked i, ii and iii contain 1 kb ladder, lambda *Hind*III Ladder and 100 bp ladder (New England Biolabs), respectively.

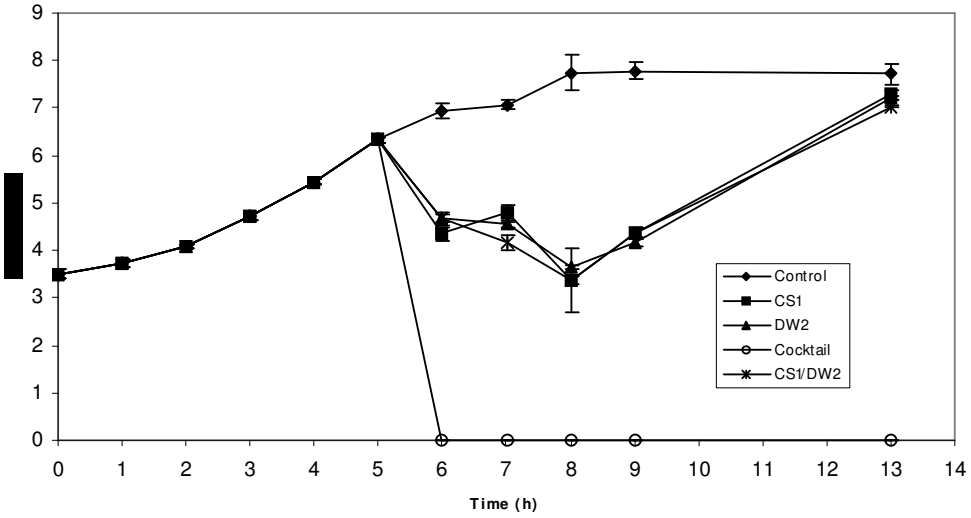


Figure 2

Bacterial challenge experiment *in vitro* against *S. aureus* DPC5246. Comparison of phage CS1, DW2, both in combination and a phage cocktail of phage CS1, DW2 and K to lyse the indicator strain *S. aureus* DPC5246. Challenge test was carried out at 37°C. Time of phage addition is indicated by an arrow. Phage and phage combinations were added at an MOI of 10. Values shown are the means +/- standard deviations.

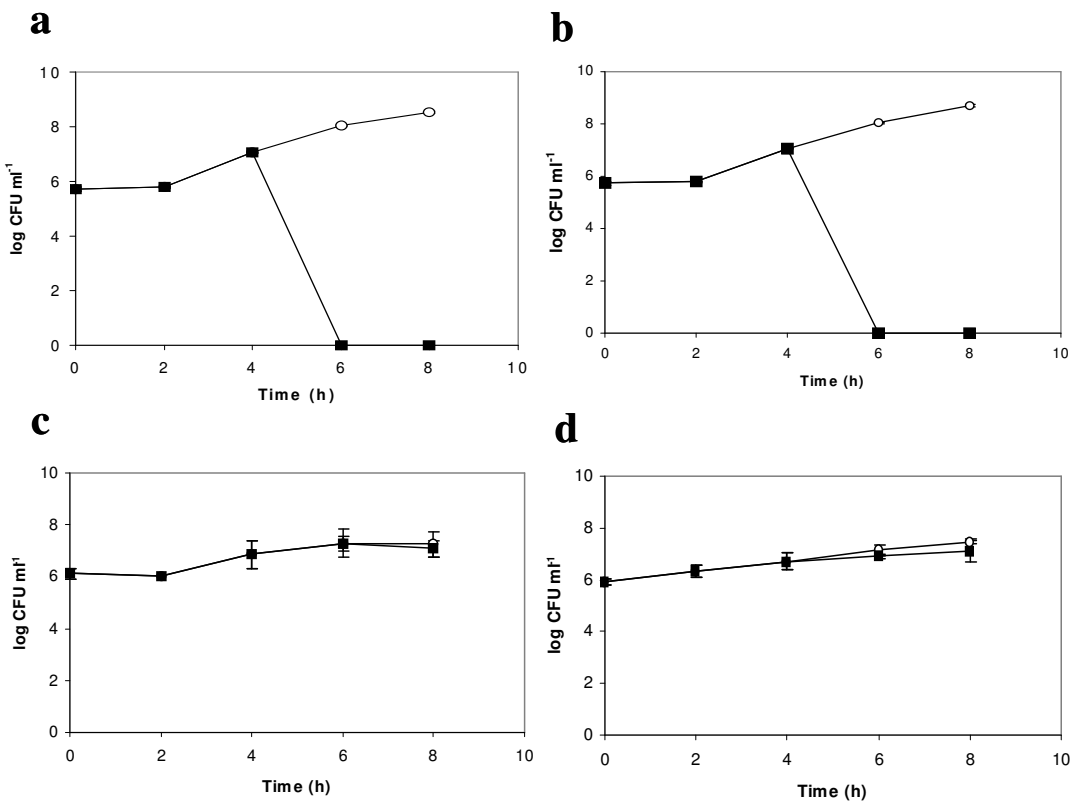


Figure 3

Challenge with phage K against *S. aureus* DPC5645 at 37°C in (a) BHI; (b) HT milk; (c) raw milk**;** (d) DCS. In each case phage K was added to *S. aureus* DPC5645 when the culture had reached a titre of approximately 10^7 CFU ml^{-1}. ○ DPC5645 without phage K; ■ DPC5645 with phage K. Error bars indicate standard error of the mean.

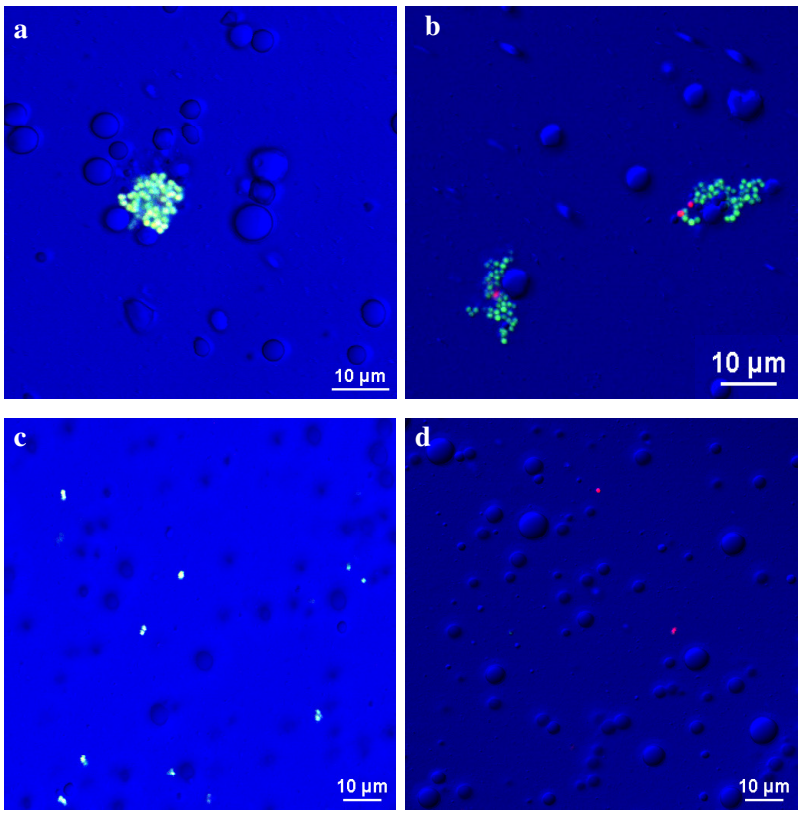


Figure 4

Confocal Scanning Laser Microscopy (CLSM) micrographs of raw milk and HT milk containing *S. aureus* DPC5246 stained with LIVE/DEAD *Bac*light viability stain. Live cells have stained green and dead cells red. (a) *S. aureus* DPC5246 in raw milk; (b) *S. aureus* DPC5246 in raw milk challenged with phage K; (c) *S. aureus* DPC5246 in HT milk; (d) *S. aureus* DPC5246 in HT milk challenged with phage K. Micrographs c and d were taken 60 minutes post phage infection.

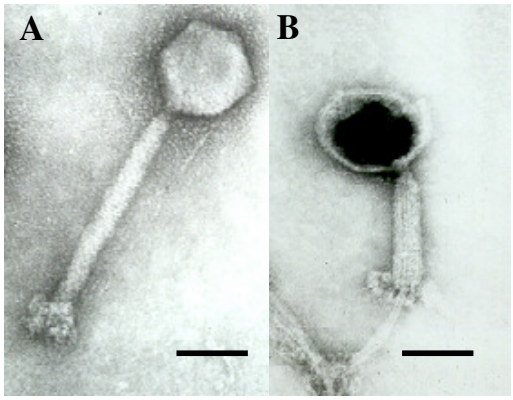


Figure 5

Electron micrograph images of phage K negatively stained with 1% uranyl acetate. (A) Phage K with contractile tail. (B) Phage K with tail contracted. Scale bar, 100 nm.

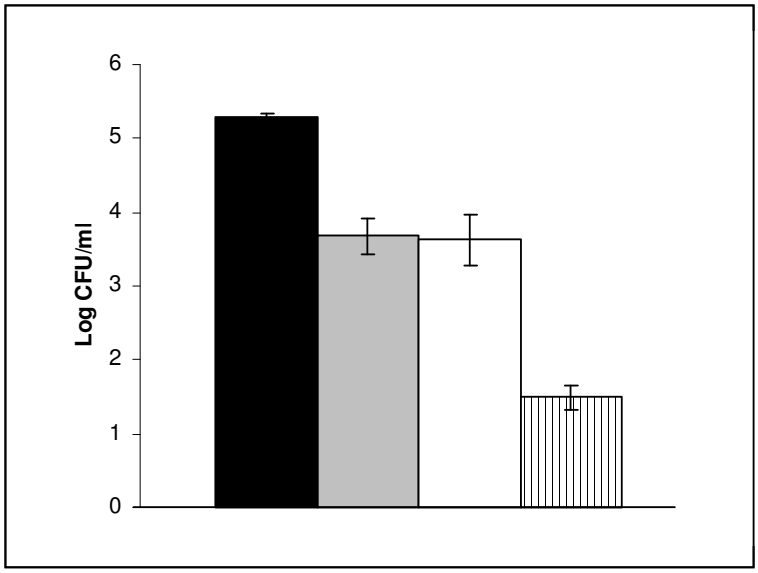


Figure 6

Graph of phage K hand wash challenge against *Staphylococcus aureus* DPC5246. Black bar, bacterial numbers in the original suspension; grey bar, unwashed control; white bar, washed control (washed with Ringers containing no phage); hatched bar, titre after washing in Ringers containing phage. Values shown are the means +/- the standard deviation.

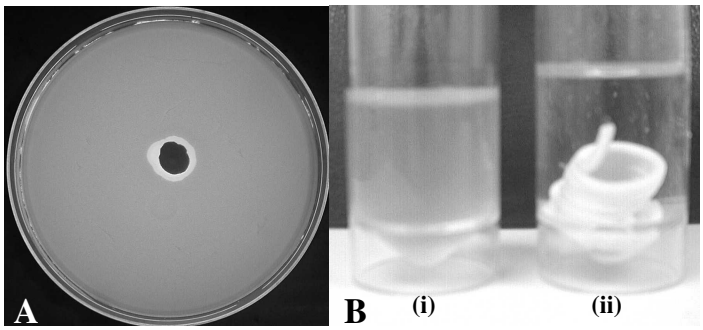
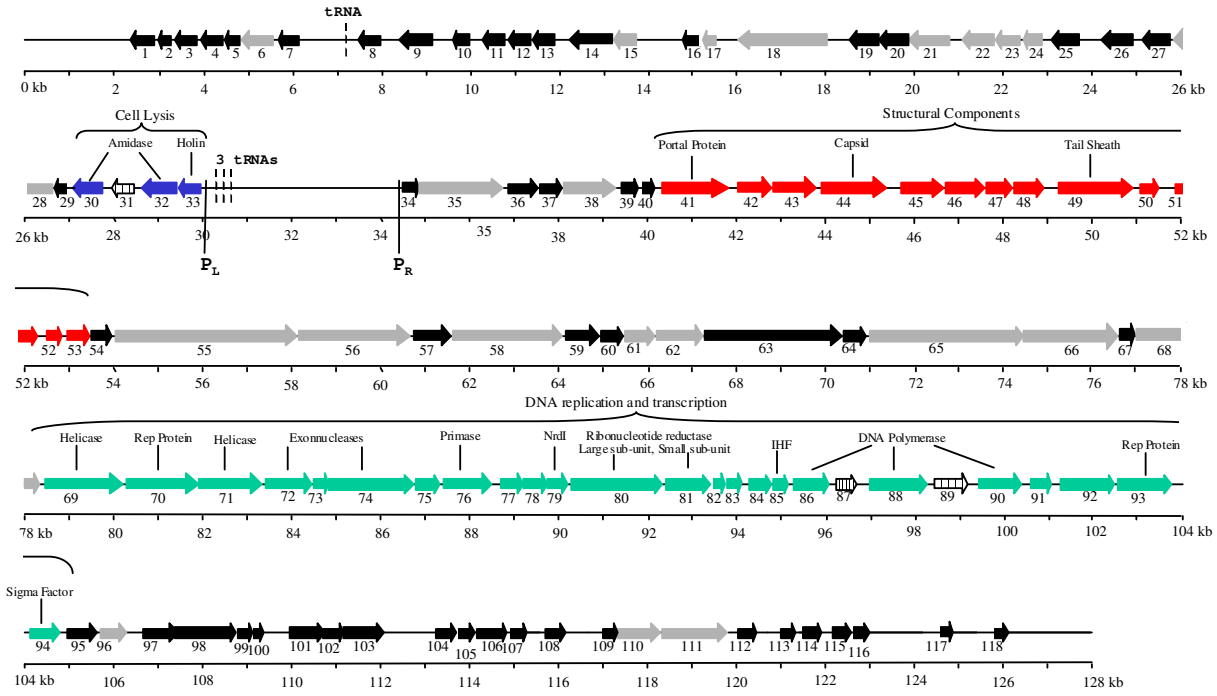


Figure 7

Activity of phage cream. (A) Activity of cream on an agar plate overlaid with the indicator strain DPC5246. (B) Activity of phage cream in BHI broth. (i) Control broth contain DPC5246 and no phage cream; (ii), broth containing DPC5246 and phage cream.



P_L 29,996 ACCGACCTACTGTTATATTATTGTTAGAAATAAATATAATAGAAA**GGTCGG**TTTTTTA**ATG** 29,935

P_R 34,448 AATTAACAAGAAAAAGTTAGAAGAAGAGGATACAAGAAAATATATAGCT**GATGGG**TTT**ATG** 34,509

Figure 8.

ORF organisation of Phage K. ORFs 1 to 118 are indicated by arrows; the numbering corresponds to that in Table 1. Blue arrows, putative lysis module; red arrows, the structural module; green arrows, DNA replication and transcription module; grey arrows, proteins with a putative function; black arrows, hypothetical proteins. Arrows with black vertical lines indicate three intron-carried ORFs. Arrows are roughly drawn to scale. Vertical lines mark two putative promoters. L and R, direction of transcription (left or right). Start codons and ribosome binding sites are indicated in boldface and putative – 10 and – 35 sites are underlined. Dashed vertical lines represent the position of four putative tRNAs.

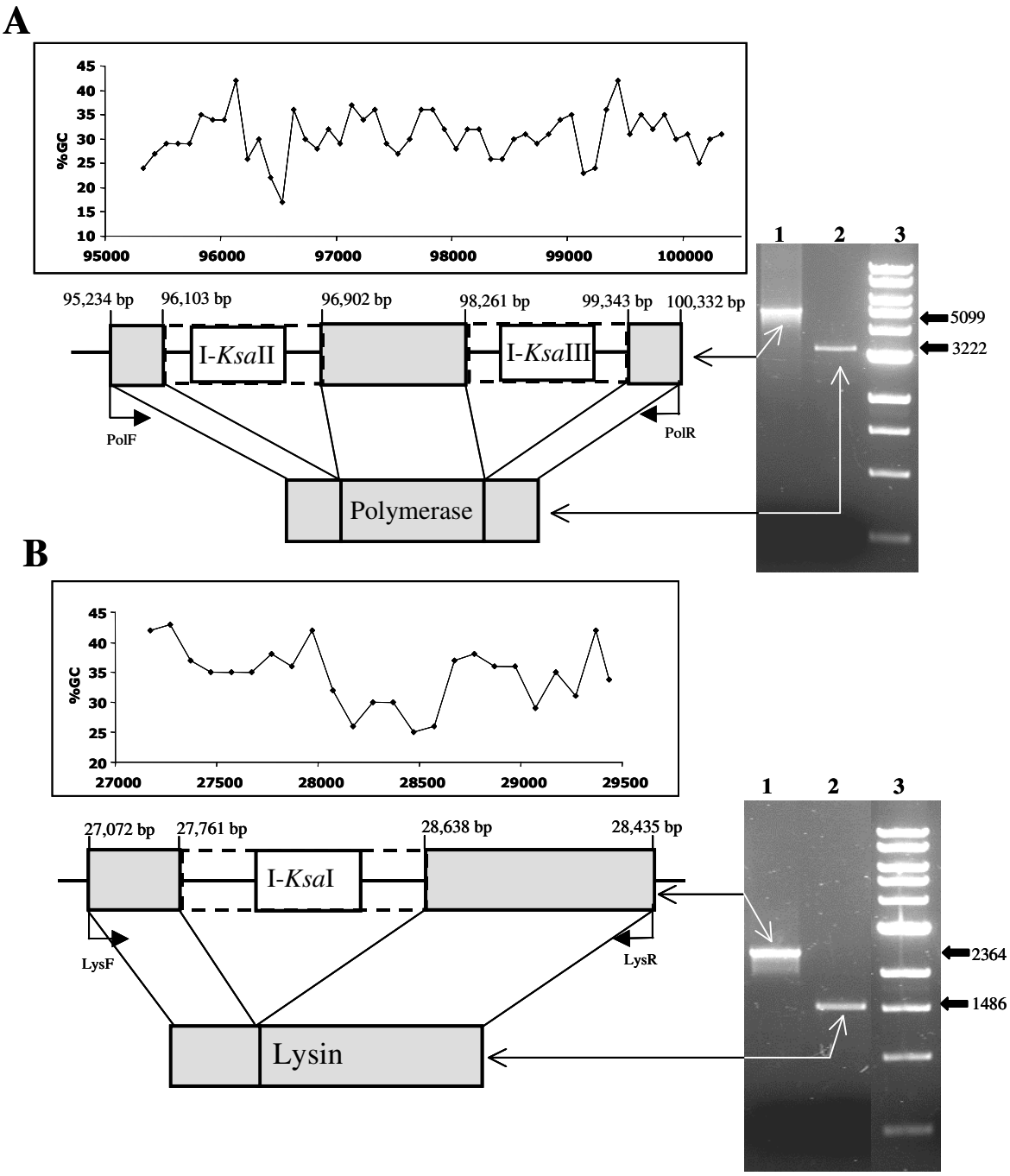
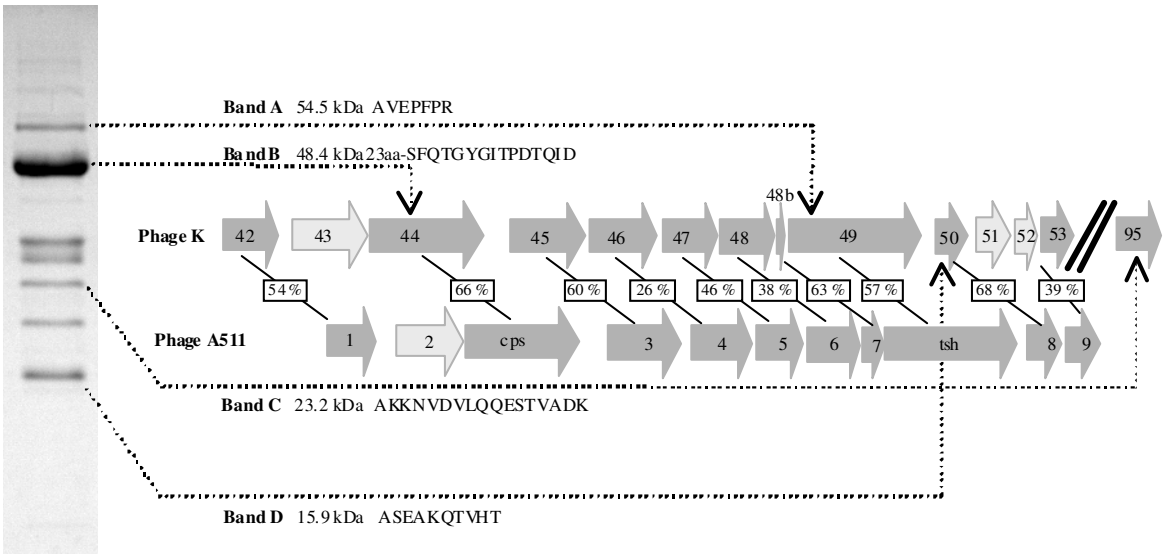

A
%GC
95000
96000
97000
98000
99000
100000
95,234 bp
96,103 bp
96,902 bp
98,261 bp
99,343 bp
100,332 bp
I-KsaII
I-KsaIII
PolF
PolR
Polymerase
1
2
3
5099
3222
B
%GC
27000
27500
28000
28500
29000
29500
27,072 bp
27,761 bp
28,638 bp
28,435 bp
I-KsaI
LysF
LysR
Lysin
1
2
3
2364
1486

Figure 9.

(A) Schematic representation of phage K polymerase gene interrupted by intron DNA. Dashed lines represent intron *pol*-I2 and *pol*-I3, encoding I-*Kas*II and I-*Kas*III' respectively. The *in vivo* splicing of phage K intron DNA from the polymerase gene is illustrated on a 1 % agarose gel. Lane 1, PCR product obtained on phage K DNA using primers PolF and PolR; lane 2, PCR product obtained from cDNA of phage K using primers PolF and PolR; lane 3,1 kb ladder (New England Biolabs). Sizes of PCR products are indicated on the right in base pairs. The schematic diagram is overlaid with a graph illustrating the % G+C content in this section of the genome. (B) Schematic representation of phage K lysin gene interrupted by intron DNA. Dashed lines represent intron *lys*-I1 encoding I-*Kas*I. The *in vivo* splicing of phage K intron DNA from the lysin gene is illustrated on a 1 % agarose gel. Lane 1, PCR product obtained on phage K DNA using primers LysF and LysR; lane 2 PCR product obtained



from cDNA of phage K using primers LysF and LysR; lane 3, 1 kb ladder (New England Biolabs). Sizes of PCR products are indicated on the right in base pairs. The schematic diagram is overlaid with a graph illustrating the % GC content in this section of the genome.

Figure 10.

One-dimensional SDS-PAGE of phage K proteins stained with Coomassie brilliant blue and schematic diagram of similarities with *Listeria* phage A511. Bands A, B, C and D represent the four proteins that were N-terminal sequenced. The N-terminal sequences of each band and the ORF from the genome of phage K to which each band corresponds are shown to the right. Numbering of ORFs for phage A511 corresponds to that in reference 23. Dark grey arrows represent ORFs with a corresponding ORF in each phage and light grey arrows represent ORFs that have no corresponding ORFs between phage K and A511. Percent identities shared between ORFs of Phage K and A511 are indicated in boxes in the schematic diagram.