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ORIGINAL ARTICLE

# Susceptibility of *Staphylococcus epidermidis* planktonic cells and biofilms to the lytic action of staphylococcus bacteriophage K

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## Abstract

**Aims:** To evaluate differences in biofilm or planktonic bacteria susceptibility to be killed by the polyvalent antistaphylococcus bacteriophage K.
**Methods and Results:** In this study, the ability of phage K to infect and kill several clinical isolates of *Staphylococcus epidermidis* was tested. Strains were grown in suspension or as biofilms to compare the susceptibility of both phenotypes to the phage lytic action. Most strains (10/11) were susceptible to phage K, and phage K was also effective in reducing biofilm biomass after 24 h of challenging. Biofilm cells were killed at a lower rate than the log-phase planktonic bacteria but at similar rate as stationary phase planktonic bacteria.
**Conclusions:** *Staphylococcus epidermidis* biofilms and stationary growth phase planktonic bacteria are more resistant to phage K lysis than the exponential phase planktonic bacteria.
**Significance of study:** This study shows the differences in *Staph. epidermidis* susceptibility to be killed by bacteriophage K, when grown in biofilm or planktonic phenotypes.

## Introduction

*Staphylococcus epidermidis* and related coagulase-negative staphylococci are now well established as major nosocomial pathogens associated with infections of indwelling medical devices. Biofilm formation is one of the major virulence factors produced by these organisms (Vuong and Otto, 2002). *Staphylococcus epidermidis* cells within biofilms acquire enhanced protection against clearance by the immune system (Cerca *et al.* 2006) and also against antibiotic therapy (Hoyle and Costerton 1991). It has been suggested that bacteriophages could be used as therapeutic agents against biofilm infections (Sillankorva *et al.* 2004) although more studies are necessary to support this conclusion. Bacteriophages are viruses that infect and kill bacteria (Chibani-Chennoufi *et al.* 2004), are ubiquitous (Ackermann and Krisch 1997); and mostly harmless to humans (Bruttin and Brussow 2005).

Although some studies have been focused on bacteriophage therapy against *Staph. aureus* (Matsuzaki *et al.* 2003; O'Flaherty *et al.* 2005), information is still lacking on *Staph. epidermidis*, specially concerning biofilm infections. Accordingly, the lytic activity of phage K, a polyvalent antistaphylococcus phage, against planktonic and biofilm bacteria of clinical isolates of *Staph. epidermidis* was evaluated in this study.

## Materials and methods

### Bacteria, phage and growth conditions

Phage K and its host propagating strain *Staph. aureus* DPC5246 were a kind gift from Dr Aidan Coffey (Cork, Ireland). Eleven clinical isolates of *Staph. epidermidis* strains, previously characterized in terms of biofilm formation ability (Cerca *et al.* 2005c), were used to test the phage host range. Tryptic soy broth (TSB) and tryptic soy agar (TSA) were prepared according to the manufacturer's instructions. Top agar was prepared by adding 0·6% agar to TSB medium. All strains were incubated in

15 ml of TSB inoculated with bacteria grown on TSA plates not older than 2 days, and grown overnight at 37°C in a shaker rotating at 130 rpm. Bacteria were then harvested by centrifugation (for 5 min at 10 500 ***g*** and 4°C), washed twice and resuspended in a saline solution (0·9% NaCl prepared in distilled water) to a concentration of $1 \times 10^9$ cells $ml^{-1}$.

### Phage propagation and enumeration

Phage propagation was performed as described previously (Sillankorva *et al.* 2004) with some modifications. Briefly, 90 ml of top agar, 1 ml of bacteria and 1 ml of phage K containing $1 \times 10^9$ plaque forming units (PFU) were added to a T-flask containing a thin layer of TSA. The T-flask was incubated for 18 h at 37°C. Phage K was removed by elution from the top agar by adding 90 ml of SM buffer (5·8 g $l^{-1}$ NaCl, 2 g $l^{-1}$ $MgSO_4 \cdot 7H_2O$, 50 ml $l^{-1}$ 1 mol $l^{-1}$ Tris pH 7·5) to the T-flask and incubated for 24 h at 4°C and 50 rpm. SM buffer was recovered and centrifuged at 10 000 ***g*** during 10 min at 4°C. Four volumes of the supernatant were added to one volume of chloroform followed by 30 s of vortexing. The organic and aqueous phases were separated by centrifugation at 4000 ***g*** during 10 min at 4°C. The aqueous phase, containing the phages, was stored at 4°C until required.

Phage enumeration was performed as described previously (Sillankorva *et al.* 2004). Briefly, 10-fold dilutions of phage solution were prepared in SM buffer. Then, 100 μl of each phage dilution were added to 100 μl of *Staph. aureus* DPC5246 resuspended in 0·9% NaCl. This suspension was mixed with 3 ml of top agar and poured onto the surface of a TSA plate. The plates were incubated for 18 h at 37°C and the number of plaques (PFU) was counted. All experiments were done in duplicate with three repeats.

### Phage host range

The ability of phage K to infect and kill *Staph. epidermidis* strains was determined in a plaque assay as described previously (O'Flaherty *et al.* 2005). Briefly, for each *Staph. epidermidis* strain, 100 μl of an overnight culture resuspended in 0·9% NaCl were mixed with top agar and poured in a Petri dish with a thin layer of TSA. When the overlay was solidified, 20 μl of phage containing $8 \times 10^9$ PFU were spotted onto the surface. Plates were air-dried and incubated at 37°C for 18 h. No lysis of the cell lawn indicated resistance to phage K; an opaque clearing indicated low susceptibility and a transparent clearing indicated high susceptibility to phage K.

### Susceptibility of planktonic cultures to phage K

For each strain susceptible to phage K, 200 μl of a cell suspension adjusted to $1 \times 10^9$ cells $ml^{-1}$ in 0·9% NaCl were added to 20 ml of TSB, and incubated at 37°C with shaking at 130 rpm, until a cell density of $2 \times 10^8$ cells $ml^{-1}$ was reached. Then, phage K was added at different multiplicities of infection (MOI) of 0, 0·1, 0·5, 1 and 5 and growth was allowed to occur during 5 h. Samples were collected at different time points, and the $OD_{640\ nm}$ was determined. Each sample was also diluted 10-fold and plated in triplicate in TSA. The plates were then incubated overnight at 37°C. This experiment was repeated three times. To determine the concentration of phage in suspension, during bacterial growth, a sample was collected every hour and phages were quantified as described above.

### Susceptibility of biofilms to phage K

For each strain susceptible to phage K, biofilms were formed as described previously (Cerca *et al.* 2005a). Briefly 50 μl of a cell suspension of $1 \times 10^9$ cells $ml^{-1}$ prepared in a 0·9% NaCl solution were added to 96 wells microtiter plate containing TSB +1% glucose (TSBG). Biofilm formation was allowed to occur during 24 h at 37°C while rotating at 130 rpm. Each biofilm was washed twice in 0·9% NaCl to remove planktonic cells. Then, $2 \times 10^8$ PFU of phage K diluted in 0·9% NaCl was added to half of the wells, and as negative control, 0·9% NaCl was added to the remaining half of the wells. The microtiter plates were incubated for 24 h at 37°C and 130 rpm. The biofilms were washed twice in 0·9% NaCl, and the total biomass of the biofilms was determined by crystal violet staining, as described previously (Cerca *et al.* 2005b), with some modifications. Briefly, biofilms were washed twice with 0·9% NaCl solution, dried in inverted position, and stained with 1% crystal violet for 10 min. The plates were washed with distilled water, and air-dried. An aliquot of 200 μl of 0·9% NaCl solution was added to each well, and the absorbance was measured in an ELISA plate reader at 570 nm. For each condition studied, three separate experiments were performed.

### Susceptibility to phage K of biofilm-grown cells *vs* planktonic cultures

*Staphylococcus epidermidis* M187 was selected to test biofilm-grown cells susceptibility to phage K. Biofilms were formed for 24 h in TSBG as described above. The biofilms were then scraped from the surface and resuspended in 0·9% NaCl, as described previously (Cerca *et al.* 2005a). Resuspended biofilms were then vortexed for 20 s and sonicated during 5 s at 10 W, to disaggregate the bacteria

(procedure previously optimized to minimize cell disruption; Cerca *et al.* 2005c). Planktonic bacteria were grown for 24 h in TSB, in order to obtain cells in the stationary growth phase. The suspension was centrifuged at 10 000 ***g*** for 5 min and resuspended in 0·9% NaCl by vortexing for 20 s and sonication during 5 s at 10 W. Next, both suspensions were diluted in a nutrient-poor medium (10% TSB diluted in 0·9% NaCl) to an $OD_{640}$ of 0·4, that was equivalent to $\sim 2 \times 10^8$ cells $ml^{-1}$, as determined by microscopic counting using a Neubauer chamber. Phage K at a MOI of one was added to each suspension, and the reduction of $OD_{640}$ was monitored during 5 h, when compared with a control without phage. This experiment was repeated three times with triplicates.

## Results

### Susceptibility of planktonic cultures to phage K

Only one of the 11 strains used was not susceptible to phage K (strain M129). Considering the remaining strains, four were highly susceptible to phage K (strains M187, FJ6, JI6, LE7), as determined by the transparent halo of the cell lawn, while the lysis of the cell lawn of the other six strains was not so evident (strains 9142, 9142-M10, IE75, IE186, IE214, PE9; data not shown). The lytic activity of phage K against planktonic cultures of *Staph. epidermidis* strains M187 and PE9 in the exponential phase of growth is illustrated in Fig. 1. These two strains are representative of the susceptibility response to phage K lysis: *Staph. epidermidis* M187, is highly susceptible to phage K and *Staph. epidermidis* PE9 exhibits a low susceptibility. The two strains also have similar biofilm formation abilities, as determined previously (Cerca *et al.* 2005c). The other highly susceptible strains demonstrated similar behaviour to strain M187 and the remaining low susceptible strains demonstrated the same behaviour as strain PE9 (data not shown).

*Staphylococcus epidermidis* M187 suffered about four $Log_{10}$ CFU $ml^{-1}$ reduction of the cell titre in <2 h, with a MOI of five or one. Even with a MOI as low as 0·1, after 5 h a three $Log_{10}$ CFU $ml^{-1}$ reduction of the cell titre was observed which corresponds to 99·90% of *Staph. epidermids* M187 cells lysis. The lytic performance of phage K against the less susceptible strains was not so high, except when using a high MOI. Nevertheless, at this high MOI and 1 h after phage challenging, bacteria started to grow again.

### Susceptibility of biofilms to phage K

To determine the action of phage K in *Staph. epidermidis* biofilms, the biofilm forming susceptible strains were grown in TSBG in microtiter plates for 24 h, after which

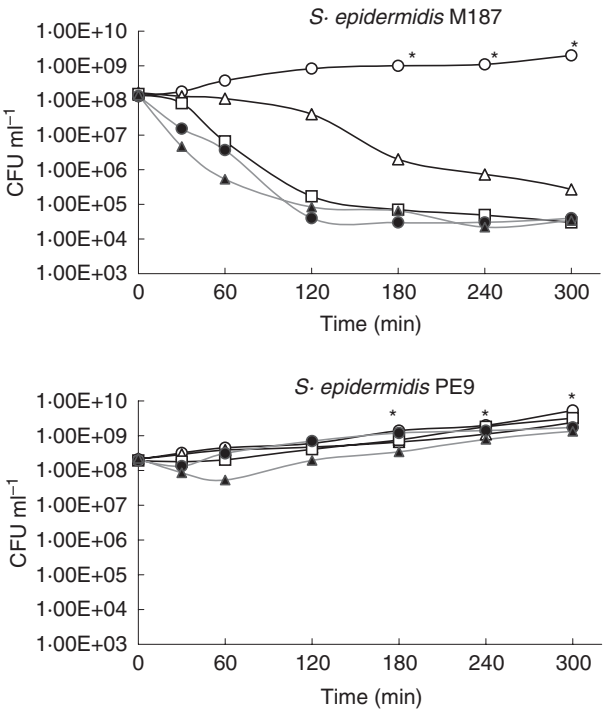


**Figure 1** Kill curves of exponential growth phase planktonic *Staph. epidermidis* M187 and PE9 by phage K at different multiplicity of infection (MOI): 0 (○), 0·1 (△), 0·5 (□), 1 (●) and 5 (▲). *At these time points, macroscopic clusters of cells were observed, suggesting that the suspensions contained more cells than were detected by plating.

biofilms were challenged with phage K. In most strains, the biomass reduction of the biofilms compared with the controls was evident (Fig. 2). Interestingly, even with the strains that were considered less susceptible to the phage in the planktonic assays, especially IE186, IE214 and also PE9 (although in lesser extent) the biomass of biofilms was also significantly reduced (statistically determined by paired samples *t*-test, $P < 0{\cdot}05$) when compared with the control.

### Susceptibility to phage K of biofilm-grown cells *vs* stationary growth planktonic cultures

To compare planktonic and biofilm-grown cell susceptibilities to phage K, the lytic assays were performed using strain M187 planktonic cells at the stationary growth phase (after 24 h of growth) and 24 h biofilm-grown cells, in a low-nutrient medium to slow down the growth rate of cells and to preserve the biofilm phenotype for the longest possible time.

Figure 3 presents the results of the effect of phage K at a MOI of one, in biofilm grown cells and also in planktonic stationary phase cells. Biofilm grown cells and stationary phase planktonic cells demonstrated similar susceptibility to phage K. It was interesting to notice that

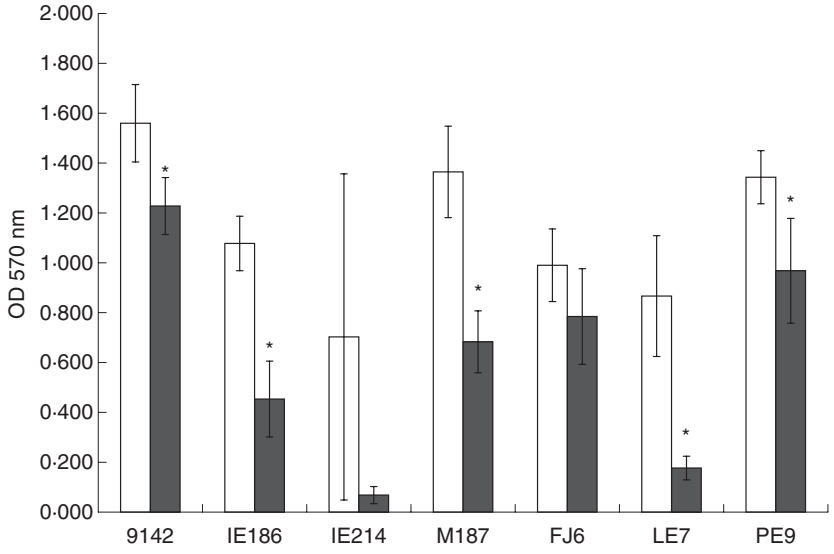


**Figure 2** Reduction of biofilm biomass after 24 h of challenge with $2 \times 10^8$ PFU of phage K. White bars represent control biofilms without phage and dark bands represent biofilm infected with phage.*Significant reduction in biomass compared with control (light bands; paired samples *t*-test, $P < 0{\cdot}05$).

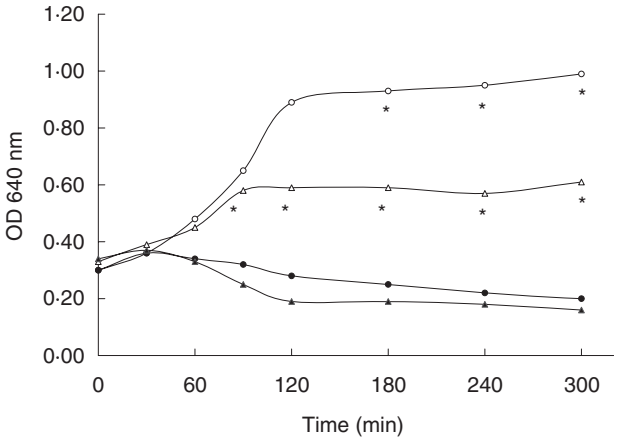


**Figure 3** Kill curves of *Staph. epidermidis M187* in different growth stages: stationary phase bacteria (control – ○, with phage – ●), or biofilm grown bacteria (control – Δ, with phage – ▲). *At these time points, macroscopic clusters of cells were observed, suggesting that there was some cell growth, even without OD increase.

the biofilm-grown cells started to aggregate relatively quickly, and after 90 min of phage challenging, no more cell growth was detected (as determined by no increase of the OD of the cell suspension), although macroscopic cells clusters were detected. Cell clusters were also detected in the planktonic culture, but only after 180 min of growth.

## Discussion

Although relatively large numbers of phages are found in biofilm-related environments, little information exists regarding the role of phages in biofilms (Sutherland *et al.* 2004), specially in *Staph. epidermidis* biofilms. Phage K has been previously described as a polyvalent phage, with the ability to infect and kill nine different species of *Staphylococcus*, including *Staph. epidermidis.* (O'Flaherty *et al.* 2005). However, the host range of phage K in *Staph. epidermidis* clinical isolates was still not evaluated. In the present study phage K was found to be effective against most of the clinical isolates previously characterized in terms of biofilm formation abilities and susceptibilities to antibiotic therapy (Cerca *et al.* 2005a). To better compare the efficacy of phage K against biofilm infections, kill experiments were performed using both the highly and poorly sensitive strains to phage K.

Planktonic cells of strain M187 were highly susceptible to phage K. A fast decrease in the number of viable cells was observed even when low concentrations of phage were used. Conversely, planktonic cells of PE9 were only susceptible to a very high phage concentration (Fig. 1). The release of new phages is expected after cell lysis in a normal lytic cycle, increasing the phage titre. This fact was only observed when M187 was the host strain (data not shown).

When phage K was tested against a 24 h biofilm of either the highly susceptible strain M187 or the poorly susceptible strain PE9, no biomass reduction was observed 5 h after phage challenge (data not shown). However, 24 h after the phage challenge, a significant reduction of the biomass of both M187 and PE9 biofilms was detected, although less pronounced for PE9 biofilms. This result suggests that phages lyse biofilms slower than planktonic cultures.Sillankorva *et al.* (2004) also showed that biomass reduction after phage challenge is faster in planktonic cultures than in biofilms of *P. fluorescens*, but nevertheless the total biomass reduction was equivalent in both planktonic and biofilm cultures. Previous studies have demonstrated that phages are able to penetrate the

biofilm matrix (Hughes *et al.* 1998; Hanlon *et al.* 2001), therefore the slower lytic performance of phages against biofilms is probably due to the physiological state of biofilm cells, and not to diffusion barriers.

In order to better compare the phage K lytic action against planktonic and biofilm cells of *Staph. epidermidis* M187, the same concentration of planktonic and biofilm cells were used in the assays. Moreover, the planktonic cells used were grown in a low nutrient medium and harvested at the stationary growth phase and the biofilms were dispersed and sonicated in order to obtain a suspension of biofilm-grown cells. Both planktonic and biofilm-grown cells demonstrated low rates of killing by phage K (Fig. 3). Interesting was the fact that biofilm-grown cells started to aggregate 90 min after the beginning of the experiment. This was also observed in planktonic cells but only after 180 min after the beginning of the experiment. This can be explained by the presence of poly-*N*-acetylglucosamine (PNAG) that can act as the intercellular adhesion in *Staph. epidermidis* (Heilmann *et al.* 1996) and that is present in higher concentrations in biofilm bacteria than in planktonic bacteria (Cerca *et al.* 2006).

Also interesting was the fact that planktonic cells in exponential growth phase were much more sensitive to phage K lysis than when in the stationary growth phase. This effect was previously demonstrated on *P. fluorescens* planktonic cultures (Sillankorva *et al.* 2004). It seems that biofilms are slowly killed by phage K, not due to a specific biofilm phenotype, but probably due to the low metabolic activity of biofilm cells (Costerton 1995; Beveridge *et al.* 1997). Nevertheless, phages might present an alternative to antibiotic therapy, since although slower than with planktonic populations, phages can also kill biofilm bacteria.

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