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Bacteriophage Φ S1 Infection of *Pseudomonas fluorescens* Planktonic Cells *versus* Biofilms

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This communication focuses on the efficacy of a specific lytic phage, phage Φ S1, as a control agent of *Pseudomonas fluorescens* biofilms. The effect of phage infection temperature and the host growth temperature were evaluated. The results obtained showed that the phage infection process was temperature dependent and that the optimum temperature of infection of planktonic cells and biofilms was 26°C. At this temperature, bacteriophage Φ S1, at a multiplicity of infection (MOI) of 0.5 infected both planktonic cells and biofilms causing a biomass reduction of about 85% in both cases.

Keywords: planktonic *vs* biofilm; *Pseudomonas fluorescens*; biofilm control; phage Φ S1

INTRODUCTION

Biofilms have a detrimental impact in the food industry, which ultimately impacts on the quality of the product. Several authors have reported that the nature of biofilm structure and the physiological attributes of biofilm organisms confer an inherent resistance to biocides, thereby rendering biofilm eradication difficult (Gilbert *et al*., 1997; Donlan & Costerton, 2002; Allison, 2003). Mechanisms such as (i) the delayed penetration of the chemical agents through the biofilm matrix, (ii) the reaction of biocides with matrix components (Simões *et al*., 2003), (iii) the altered growth rate of biofilm organisms, and (iv) other physiological changes due to the biofilm mode of growth have been described as factors influencing biocide resistance (Donlan & Costerton, 2002). Thus, the development of new approaches to eradicate biofilms from industrial environments is of the utmost importance.

Lytic bacteriophages (phages) by their nature can be seen as good candidates for biofilm control. Phages are highly specific, affecting only the target bacterium and thus do not interfere with the existing microbial community. This is especially attractive for dairy industries, in which other bacteria like *Lactobacillus* sp. are important for food processing and should not be eliminated. In addition, phages are non-toxic to humans, animals and plants and can be effective against biocide resistant bacteria. Furthermore, phage production is simple and relatively inexpensive. Although veterinary and medical applications of phages against antibiotic resistant bacteria have been intensively described (Barrow, 2001; Sulakvelidze *et al*., 2001), there are few studies on the interaction of phages with biofilms (Hughes *et al*., 1998; Corbin *et al*., 2001; Hanlon *et al*., 2001; Tait *et al*., 2002). Cells in a biofilm are in close contact, which can enhance phage replication compared to planktonic cells (Wiggins & Alexander, 1985).

In this communication, the control of *Pseudomonas fluorescens* biofilms by a lytic phage Φ S1 was assessed. This bacterium is particularly problematic in dairy industries as it causes milk spoilage due to the production of proteolytic enzymes. Moreover, *P. fluorescens* biofilms are difficult to eradicate using the traditional chemical biocides (Simões *et al*., 2003). The effect of biofilm growth temperature and infection temperature in phage performance was also evaluated.

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MATERIALS AND METHODS

Bacteria, Bacteriophage and Media

Pseudomonas fluorescens ATCC 27663 and the respective phage Φ S1 from the American Type Culture Collection (ATCC) were used in this study. According to ATCC both phage and bacterium were isolated from soil samples.

Nutrient broth medium (NBM) contained 10 g l^{-1} nutrient broth, 1 g l^{-1} glucose, 1.45 g l^{-1} $K_2HPO_4.3H_2O$ and 0.49 g l^{-1} KH_2PO_4. Nutrient broth agar (NBA) contained NBM with the addition of 1.5% (wt/vol) agar. Nutrient broth soft agar (NBSA) contained 10 g l^{-1} nutrient broth, 2% (wt/vol) glucose, 1.45 g l^{-1} $K_2HPO_4.3H_2O$, 0.49 g l^{-1} KH_2PO_4 and 0.6% (wt/vol) agar. The phage buffer used for phage dilution and preservation contained 0.73 g l^{-1} Trizma base, 0.5 g l^{-1} of gelatine, and 2.5 g l^{-1} of $MgSO_4.7H_2O$.

Bacteriophage Propagation and Purification

For phage propagation, 30 ml of NBSA were added to a mixture of 1 ml of an overnight culture of *P. fluorescens* and 1 ml of phage suspension containing 10^3 plaque forming units (PFU) ml^{-1}. This mixture was poured onto a T-flask containing a thin layer of NBA. The T-flask was incubated for 7 h at 26°C. Phage Φ S1 was removed by elution from the soft agar overlays by adding 30 ml of phage buffer to the T-flask. The flask was incubated for 24 h at 4°C to allow the phage to diffuse into the liquid phase. This phase was recovered, and sodium chloride to a final concentration of 1 M was added. The solution was stored on ice for 1 h after which bacterial debris were removed by centrifugation at 11,000 × g for 10 min at 4°C. Polyethylene glycol 10,000 was added to the recovered liquid phase to a final concentration of 10% (wt/vol) and was slowly dissolved by stirring. The mixture was left overnight at 4°C. The precipitated phage particles were recovered by centrifugation at 11,000 × g for 10 min at 4°C. The pellets were resuspended in phage buffer, and chloroform was added to the solution. This was followed by vortexing for 30 s. The organic and aqueous phases were separated by centrifugation at 3000 × g for 15 min at 4°C. The aqueous phase, containing the phages, was stored at 4°C until required.

Bacteriophage Enumeration

Phage enumeration was done according to the soft agar overlay technique described by Adams (1959). Briefly, the method consists in diluting the sample, containing the phages to be enumerated, in phage buffer. Then 100 μl of a determined phage dilution were added to 100 μl of overnight grown *P. fluorescens*. This suspension was mixed with 3 ml of NBSA and poured onto the surface of a NBA plate. The plates were incubated for 18 to 24 h at 26°C and the number of plaques and single halos (PFU) were counted. All the analyses were replicated three times.

Cell Lysis Assessment

The rate of cell lysis after phage infection was evaluated as the rate of decrease in absorbance and the rate of ATP increase. ATP measurement is especially useful to follow cell lysis in biofilms where measurement of absorbance cannot be employed. It is also a suitable method when the rate of cell growth overcomes the rate of cell lysis, which results in an increase in the optical density. A linear relation was obtained between ATP increase and decrease in absorbance after phage infection of *P. fluorescens* cells. The amount of ATP released is not dependent on cell growth temperature or on cell growth phase. This was evaluated by measuring the ATP released after disruption by sonication (60s, 60W, Vibra Cell Sonicator) of cells grown at 4°C, 26°C, and 37°C.

The ATP was measured by mixing 100 μl of sample with 100 μl of a 25 fold dilution mixture of luciferin and luciferase (Sigma FL-AAM). The light transmission was then measured with a bioluminometer (Lumac, Biocounter M 25000).

Biofilm Formation

P. fluorescens biofilm was formed on 2 × 2 cm^2 inox plates placed in a 6 well microtiter tray. Nine ml of *P. fluorescens* culture with an absorbance of approximately 0.75 were added to each well containing an inox plate and the different trays were incubated at 4°C, 26°C and 37°C for 12 h at 130 rpm. After incubation the suspension was removed from the trays and fresh NBM was added. The biofilm was formed over 5 d with a change of medium every 12 h.

Determination of the Number of Cells in the Biofilm

A biofilm sample of 0.5 g wet weight was extracted with Dowex resin according to the method described by Azeredo *et al.* (2003) in order to separate the EPS matrix from the cells. The dry weight of the cell portion was determined after drying for 24 h at 105°C. The corresponding number of cells was obtained through a calibration curve of dry weight *vs* number of cells. The cells were enumerated using a Neubauer chamber. The dry weight of the cellular portion of 0.5 g (wet weight) of the biofilm was 0.0125 ± 0.0002 g, corresponding to $1.54 \times 10^{11} \pm 0.2 \times 10^{11}$ cells.

Infection Experiments on Planktonic Cells

The effect of growth temperature

P. fluorescens were grown at 4°C, 26°C and 37°C. The cells were harvested at the exponential growth phase by centrifugation at 5000 × g for 10 min at 4°C, and suspended in fresh NBM, at the temperature at which they were grown (4°C, 26°C and 37°C). Equal volumes of phage, NBM and a suspension of *P. fluorescens* were mixed. The flasks were incubated at 130 rpm at 4°C, 26°C and 37°C. At defined time periods, samples were taken for ATP measurement in order to follow cell lysis. Samples for phage enumeration were centrifuged for 10 min at 9600 × g to precipitate the bacteria and the titer was determined as described above. The multiplicity of infection (MOI), the average number of phage per bacterium, used in planktonic cell assays was 0.5. The dry weight of the cell suspension before and after phage infection was measured. The assays were performed four times, with each assay done in triplicate.

The effect of growth phase

P. fluorescens planktonic cells were grown at 26°C in NBM for 24 h, 48 h and 72 h, until the exponential, stationary and decline phases, respectively were reached. These cultures were also subjected to phage infection as described previously.

Infection Experiments on Biofilm

After 5 d growth, the wells were washed in order to remove non-adhered cells and then the biofilm was removed by scraping. Portions of 0.5 g wet weight (equivalent to $1.54 \times 10^{11} \pm 0.2 \times 10^{11}$ cells) were placed in Erlenmeyer flasks. To each flask, 16 ml of NBM and 8 ml of the phage solution (9.0×10^9 PFU ml^{-1}) were added in order to obtain a MOI of approximately 0.5. A control sample was prepared containing the biofilm portion and 30 ml of NBM. Samples were taken periodically for ATP and PFU measurements. The dry weight of the biofilm before and after phage infection was measured. The assays were performed four times in triplicate.

Statistical Analysis

To compare the results of the rate of cell lysis and the rate of phage release obtained with planktonic cells and biofilms, one-way analysis of variance (One-way ANOVA) was used. Then *post hoc* testing using Tukey's test was performed to see which data were significantly different. In all the analyses performed the confidence interval used was 95%. These tests were performed using SPSS 11.5 for Windows.

RESULTS

Effect of Phage Infection Temperature (T_I) on Planktonic and Biofilm Cells of *P. fluorescens*

Figure 1 shows the number of cells lysed after phage infection of planktonic cells and biofilms, grown and infected at 26°C. Cell lysis was higher when the infection occurred in planktonic cells, although lysis began more rapidly in biofilms. As no cell lysis was observed in the control samples, it can be concluded that lysis was only due to phage infection.

The rate of cell lysis (k) was determined by the rate of $\log_{10} (N/N_0)$, in which N_0 and N are respectively the number of cells lysed before and after the exponential period of lysis. Another important parameter was the rate of phage release (PFU/t) that was calculated by $PFU/t = (PFU_{MAX} - PFU_{MIN})/t$ in which PFU_{MAX} and PFU_{MIN} are the maximum and minimum values of PFU (plaque forming units) obtained, respectively, and t the time period between these two values. The parameters obtained after phage infection of planktonic cells and biofilms, at each infection temperature (T_I) assayed, are listed in Table I. At 26°C the rates of cell lysis and phage release were greater in planktonic cells than in biofilms; the reverse happened at 37°C, where the infection was higher in biofilms than in planktonic cells ($P < 0.05$). These differences were not statistically significant when infection occurred at 4°C ($P < 0.05$).

The optimal infection temperature was 26°C, and at this temperature the rates of phage release (PFU/t) after infection of both biofilms and planktonic cultures were greater than those obtained at 4°C and 37°C ($P < 0.05$, Table I).

Effect of *P. fluorescens* Growth Phase on Phage Infection

The rates of phage release and cell lysis of bacteria at different growth phases were statistically different ($P < 0.05$, Table II). Cells at the exponential growth

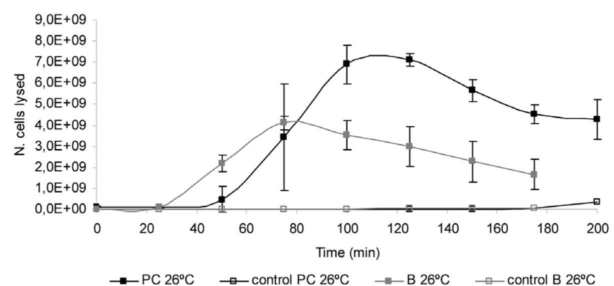

FIGURE 1 Number of *P. fluorescens* cells lysed in planktonic cells (PC) and biofilms (B) grown and infected at 26°C and the respective control samples. Both the control PC and B samples had zero cells lysed over 180 min. Error bars = SDs.

TABLE I Rate of cell lysis (k) (± SDs) and rate of phage release (PFU/t) (± SDs) following phage infection of *P. fluorescens* planktonic (PC) and biofilm (B) at different infection temperatures (T_I) using an initial MOI of 0.5

T_I (°C)	Cells	k (min^{-1})	PFU/t × 10^{-8}
4	PC	0.009 ± 0.003	3.22 ± 0.26
	B	0.012 ± 0.001	3.48 ± 0.12
26	PC	0.024 ± 0.008	16.90 ± 0.28
	B	0.020 ± 0.002	4.60 ± 2.87
37	PC	0.011 ± 0.003	0.12 ± 0.05
	B	0.017 ± 0.001	0.49 ± 0.23

TABLE II Rate of cell lysis (k) (± SDs) and rate of phage release (PFU/t) (± SDs) following phage infection at 26°C of *P. fluorescens* cells at different growth phases

Growth phase	k (min^{-1})	PFU/t × 10^{-8} (PFU ml^{-1} min^{-1})
Exponential	0.024 ± 0.008	16.90 ± 0.28
Stationary	0.017 ± 0.002	7.45 ± 0.18
Decline	0.012 ± 0.001	1.96 ± 0.37

phase were more susceptible to phage infection than cells at the stationary and decline phases.

Effect of Growth Temperature (T_G) of *P. fluorescens* Planktonic and Biofilm Cells on Phage Infection

The greatest phage efficacy was obtained when both planktonic cultures and biofilms were grown at 26°C. The rates of phage release obtained at this growth temperature were greater than those obtained at 4°C and 37°C ($P < 0.05$) (Table III).

Comparing the infection of planktonic cultures with that of biofilms, the infection parameters differed significantly only when the growth temperature was 26°C ($P < 0.05$). In this case, the rates of phage release and cell lysis were greater in planktonic cells than in biofilms (Table III).

In order to evaluate the efficacy of phage as a control agent of biofilms *vs* planktonic cells at 26°C, the percentage of biomass decrease during the course of infection was determined by measuring the dry weight before and after the infection period. It must be stressed that the same MOI (0.5) was used for planktonic cells and for biofilms. The biomass removal percentages obtained after 200 min for planktonic cells was 86.27% ± 0.88 and for biofilms 84.27% ± 0.42. These percentage values are approximately the same for biofilms and planktonic cells. *P. fluorescens* biofilms are characterized by having a thick polymeric matrix (Azeredo *et al*., 2003; Simões *et al*., 2003). The high efficiency obtained in biofilms indicate that phage was not affected by cells being embedded in a polymeric matrix. Furthermore, the high biomass decrease of biofilms following phage infection suggests that both cells and exopolymers were disrupted and solublized by the phage.

Biofilm infection at 26°C was monitored at different stages and the corresponding photographs are presented in Figure 2.

DISCUSSION

In natural environments, bacteria, including *P. fluorescens*, exist mainly in the form of biofilm. Although phage infection of planktonic cells can be easily demonstrated in environments such as natural aquatic environments (Fuhrman & Suttle, 1993; Wommack & Colwell, 2000), wastewater treatment (Muniesa & Jofre, 1998; Mocé-Llivina *at al*., 2003) and industrial facilities (Brüssow *et al*., 1994; Kiliç *et al*., 1996), there are only a few studies concerning phage infection of biofilms. The biofilm mode of growth is distinct from planktonic growth as in biofilms individual bacteria are encased in a matrix of extracellular polymeric substances (EPS) (Allinson, 2003). Bacteria are not distributed uniformly throughout a biofilm but rather aggregate into microcolonies (Lawrence *et al*., 1991; Sternberg *et al*., 1999). Due to limited access to nutrients and oxygen in the interior of the microcolonies cell growth rate can be lower (Costerton *et al*., 1987; Fletcher, 1991; Anwar *et al*., 1992), with the most metabolically active cells situated on the periphery of each microcolony (Sternberg *et al*., 1999; Azeredo & Oliveira, 2000).

Phage infection processes depend on the intracellular resources of their hosts, which depend in turn on the physiological state of the host bacteria.

TABLE III Rate of cell lysis (k) (± SDs) and rate of phage release (PFU/t) (± SDs) following phage infection of *P. fluorescens* planktonic cells (PC) and biofilms (B) grown at different temperatures (T_G) and infected at 26°C

T_G (°C)	Cells	k (min^{-1})	PFU/t × 10^{-8}
4	PC	0.014 ± 0.008	4.29 ± 0.07
	B	0.019 ± 0.003	2.17 ± 0.04
26	PC	0.024 ± 0.008	16.9 ± 0.28
	B	0.021 ± 0.002	4.60 ± 0.29
37	PC	0.010 ± 0.003	0.35 ± 0.01
	B	0.009 ± 0.001	0.11 ± 0.02

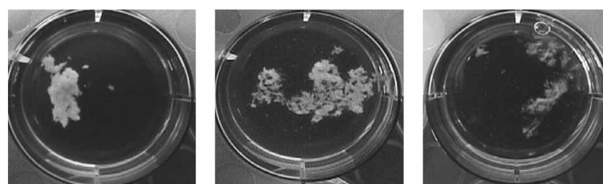

FIGURE 2 Photographs of portions of biofilm at different stages of infection at 26°C. 1 = time 0; 2 = 90 min infection; 3 = 200 min infection.

Some authors have reported that the faster the host grows the faster the phages will replicate (Hadas *et al*., 1997). Thus, it is expected that the efficacy of a planktonic culture phage infection would be higher than that of a biofilm. The present study revealed that the infection of a planktonic culture of *P. fluorescens*, at the optimal growth and infection temperature (26°C) resulted in a sharp increase of cell lysis (Figure 1), whereas the rate of cell lysis in biofilms was significantly lower. Furthermore, the rate of phage production in a planktonic culture was significantly higher than that in biofilms at 26°C, using the same initial MOI (Table I). The rate of phage release following an infection of *P. fluorescens* cells at different growth phases revealed that the infection of cells at the exponential phase yielded a greater rate of phage release than stationary and decline phase cells, respectively (Table II). The existence in biofilms of cells at different growth stages can explain the lower rate of phage production in comparison to that obtained after the infection of a planktonic culture. Another possible explanation can be the slow access by the phage into the existing bacteria on account of the biofilm architecture, as reported by Hanlon *et al*. (2001). Although phage infection and replication was faster in a planktonic culture, after 3 h of infection both planktonic cells and biofilms showed the same biomass reduction of about 85%.

In industrial environments biofilms are formed at different process temperatures. Therefore, it was important to study the effect of biofilm infection temperature, as well as the influence of the host growth temperature on the efficacy of phage infection. The results obtained concerning the effect of infection temperature revealed that the optimum phage infection temperature of biofilms and planktonic cells was 26°C. At this temperature, the rates of cell lysis and phage release presented the greatest values (Table I). When phage infection occurred at 4°C and 37°C, the rate of phage release was smaller, both in assays performed with biofilms and planktonic cells. This result suggests that the rate of phage replication decreases when infection occurs at temperatures below and above the optimal, which is probably due to an inhibition of the host cell protein synthesizing system.

The rate of phage release is dependent on the host growth temperature (Table III). This may be related to physiological changes in *P. fluorescens* grown at different temperatures that reflect the DNA, protein or RNA contents or even cell size (Araki, 1991; Guillou & Guespin-Michel, 1996; Hadas *et al*., 1997; You *et al*. 2002). The effect of growth temperature of biofilms and planktonic cells on phage infection was similar. In all assays performed, the rate of phage release was greater in planktonic cells than in biofilms. Again, different metabolic activities of cells within the biofilm can explain these results.

A considerable drawback to the use of phages as bacterial control agents is the emergence of phage-resistant bacteria. This phenomenon has been reported in many studies related to phage therapy (Berkowitz, 1995; Tenover & Hughes, 1996). In the present case, cell growth reappeared after approximately 10 h of phage infection in all assays performed. These cells were recovered and infected again with fresh phages but the results showed that they had not acquired resistance to this phage (data not shown).

In industrial environments the elimination and inactivation of microorganisms from the surfaces is crucial. When disinfection takes place without the removal of biofilms, inactivated biofilm may provide an ideal environment for further bacterial adhesion and growth (Simões *et al*., 2003). Studies concerning chemical biocide utilization in inactivation and biofilm removal from surfaces have shown that these agents have poor removal action (< 25%). Furthermore, the damage caused to the biofilm structure was not noticeable after 30 min treatment (Simões *et al*., 2003). In the present study, phage infection of biofilms and planktonic cells at 26°C resulted in a decrease of approximately 85% in the biomass content after 200 min of infection. This indicates that phage Φ S1 efficiency was not affected by the sessile mode of growth of *P. fluorescens* in biofilms. Nevertheless, cell lysis and phage release was faster in suspended cultures than in biofilms (Table III). At 26°C phage was able to destroy the cells and also the biofilm matrix, which appeared to be completely solublized (Figure 2).

The present study shows that biofilms formed and infected with phage at 26°C can be more efficiently controlled than by using traditional chemical biocides as phage caused both cell lysis and biofilm disruption. Phage efficacy was, however, dependent on host growth and infection temperatures and, therefore, the use of phage Φ S1 as a biofilm control agent is limited to optimal conditions. For this reason, the development of alternative methods, such as combination of different phages or use of depolymerase enzymes in combination with other biocide agents (Sutherland *et al*., 2004), would be interesting. Further studies are required to verify these possibilities.

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