Pseudomonas fluorescens infection by bacteriophage ΦS1: the influence of temperature, host growth phase and media

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Abstract

The influence of host growth temperature, phase and media, together with the effect of infection temperature on bacteriophage ΦS1 infection of *Pseudomonas fluorescens* were examined. The rates of cell lysis and phage release were determined and showed that the efficacy of phage infection was optimal with host cells grown and infected at 26 °C. The host physiological state also affected these rates. Infection was dependent on the presence of cell wall proteins with molecular weights of 17.5 ± 1 and 99 ± 5 kDa.
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1. Introduction

Research on the ecology of phages is essential since phages are wide spread in the environment being important bacterial population regulatory agents. For example, in aquatic environments phages can disrupt the flow of energy and carbon within ecosystems due to cell lysis at the base of the food web [1].

In industrial environments the interest in the application of phages to control bacterial populations is increasing due to the emerging appearance of biocide-resistant bacteria [2]. Furthermore, phage-therapy is again attracting attention due to the increase of bacterial resistance to antibiotics [3,4].

The phage infection process is dependent on the physicochemical characteristics of phage genome-encoded functions and on intracellular resources of the host. These host resources depend further on the cell physiological state, which is modulated by, for example, growth media [5,6] and temperature [7,8]. Phage adsorption to the host is mediated by specific interactions of phage proteins with molecules or structures on the bacterial surface. Phages may bind to cell structures, such as flagella or capsules, or to molecules on the cell wall, such as lipopolysaccharides or proteins [9].

Pseudomonas fluorescens are psychrotrophic bacteria commonly found in water and soil, and frequently encountered in industrial environments. In soil, the presence of *P. fluorescens* is important as it is a soil improving bacterium which also contributes to the control of many plant diseases caused by fungi [10,11]. In industrial environments, such as the dairy industry, the presence of *P. fluorescens* is undesirable,

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since it is responsible for product spoilage due to the production of extracellular heat resistant lipases and proteases [12].

Pseudomonas fluorescens can be infected by the lytic phage ΦS1, a *Podoviridae* virus. This type of virus is characterised as a dsDNA virus with a head, a short and non-contractile tail and lacks any envelope.

This work focuses on the interaction between phage ΦS1 and its specific host, *P. fluorescens* strain Migula. Several parameters that influence phage infection were assessed, such as (i) growth temperature and infection temperature, (ii) host growth phase and (iii) host growth media.

2. Materials and methods

2.1. Bacteria, bacteriophage and media

Pseudomonas fluorescens Migula ATCC 27663 from the American Type Culture Collection was used for bacteriophage ΦS1 propagation and titration.

Nutrient broth media (NBM) contained 10 g nutrient broth, 1 g glucose, 1.45 g $K_2HPO_4 \cdot 3H_2O$ and 0.49 g KH_2PO_4 per litre of final volume and 15 g l^{-1} were added to the solid medium cultivation (NBA). Nutrient broth soft agar (NBSA) contained 10 g l^{-1} Nutrient broth, 2% (w/v) glucose, 1.45 g l^{-1} $K_2HPO_4 \cdot 3H_2O$, 0.49 g l^{-1} KH_2PO_4 and 0.6% (w/v) agar. The phage buffer contained 0.73 g l^{-1} Trizma base, 0.5 g l^{-1} of gelatine, and 2.5 g l^{-1} of $MgSO_4 \cdot 7$-H_2O and was used as phage dilution and preservation buffer.

2.2. Bacteriophage replication and purification

For bacteriophage ΦS1 replication 30 ml of NBSA were added to a mixture of 1 ml of an overnight culture of *P. fluorescens* and 1 ml of a phage solution (10^3 PFU/ml). 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. After incubation 30 ml of phage buffer was added and the T-flask was left 24 h at 4 °C. The liquid was recovered and sodium chloride (1 M) was added. The solution was stored on ice for 1 h, after which bacterial debris were removed by centrifugation (11,000*g*, 10 min at 4 °C). 10% (w/v) and polyethylene glycol 10,000 (PEG) was slowly added to the recovered liquid and left overnight at 4 °C. The precipitated phage particles were recovered by centrifugation (11,000*g*, 10 min at 4 °C), and the pellets were resuspended in phage buffer. Chloroform (1:3) was added to the solution and mixed for 30 s. The organic and aqueous phases were separated by centrifugation (3000*g*, 15 min, 4 °C). The aqueous phase was stored at 4 °C until required.

2.3. Bacteriophage enumeration

Bacteriophage ΦS1 enumeration was done according to the methodology described by Adams [13]. The number of phages was expressed in terms of plaque forming units (PFU). All the assays were performed in triplicate.

2.4. Infection experiments

2.4.1. Effect of temperature
Pseudomonas fluorescens grown at different temperatures were harvested at the exponential growth phase by centrifugation (7000*g*, 10 min, 4 °C) and suspended in fresh NBM. The multiplicity of infection (MOI) used was 0.5. For the different temperatures equal proportions of phage, media and host suspension were mixed and incubated at 130 rpm. Periodically, samples were taken for ATP and optical density (O.D.) measurements in order to follow cell lysis. Samples for phage enumeration were centrifuged (9600*g*, 10 min) to precipitate the bacteria.

2.4.2. Effect of growth phase
Pseudomonas fluorescens were grown at 26 °C for 24, 48 and 72 h that correspond to the exponential, stationary and decline phases. The cultures were subjected to phage infection as described previously.

2.4.3. Effect of growth media
Pseudomonas fluorescens were grown in glucose medium (GM) containing 5 g l^{-1} glucose, 2.5 g l^{-1} peptone, 1.25 g l^{-1} yeast extract, 4.3 g l^{-1} $Na_2HPO_4 \cdot 2H_2O$ and 3.75 g l^{-1} KH_2PO_4 until the exponential growth phase, followed by phage infection as described previously.

2.5. Cell lysis assessment

The rate of cell lysis after phage infection was evaluated by means of the rate of optical density decrease and the rate of ATP increase. When cells are lysed ATP is released into the extracellular solution and consequently detected using a bioluminometer. ATP measurement is useful to follow cell lysis in biofilms, where optical density measurements cannot be employed. It is also suitable when the rate of cell growth is greater than the rate of cell lysis, resulting in an increase in the optical density. A linear relation was obtained between ATP increase and O.D. decrease after phage infection of *P. fluorescens* cells ($\Delta ATP = 1.3715 \times \Delta O.D. - 0.0238$). Moreover, the amount of ATP released is not dependent on cell growth temperature and on cell growth phase. This was evaluated by measuring the ATP released after disruption by sonication (60 s, 60 W, Vibra Cell sonicator) of cells grown at 4, 26, 37 °C and of cells grown for 24, 48 and 72 h.

The ATP was measured mixing 100 μl of sample with 100 μl of a 25-fold dilution of a mixture of luciferin and luciferase (Sigma FL-AAM). The light transmission was

measured with a bioluminometer (Lumac, Biocounter M 25000).

2.6. Outer membrane protein isolation and analysis

Outer membrane proteins (OMP) were isolated according to the method described by Masuda et al. [14]. Protein concentrations were determined by the Bicinchoninic Acid Protein Assay Kit (BCA) with bovine serum albumin as standard. The outer membrane fractions obtained were subjected to sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), as reported by Laemmli [15], with 12% (w/v) acrylamide and performed at a constant current of 10 mA; after electrophoresis, the gels were stained with Coomassie blue and afterwards silver stained.

2.7. Statistical analysis

To compare the results of the rate of cell lysis and the rate of phage release, obtained with different temperatures, growth and infection media and growth phases 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 analysis performed the confidence interval used was 95%. These tests were performed using SPSS 11.5 for Windows.

3. Results

3.1. Effect of temperature

Fig. 1 presents the number of cells during the course of phage infection of *P. fluorescens* grown and infected at different temperatures where time 0 corresponds to the moment of phage addition. For all temperatures assayed with the exception of 37 °C, the number of cells decreased exponentially some min after phage incubation. The results also indicate that 26 °C was the growth and infection temperature at which the number of cells lysed was the greatest. Therefore, in order to evaluate the effect of growth temperature and infection temperature separately, cells were grown at 26 °C and infected at different temperatures (Fig. 2) and were grown at different temperatures and infected at 26 °C (Fig. 3). Comparing the infection profiles obtained in Figs. 2 and 3 with Fig. 1 it is apparent that both infection and growth temperatures influence phage performance.

A more detailed study was carried out with only three temperatures of the range of temperatures studied.

Table 1 presents the rate of cell lysis as well as other infection parameters after phage infection of cells grown and infected at different temperatures. The rate of cell lysis (k) was determined by the slope 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. The rate of phage release (PFU/t) was calculated by $(\text{PFU}_{\max} - \text{PFU}_{\min})/t$, in which the parameter t corresponds to the time period between the minimum and maximum values of PFU observed. The latent-period (L) is defined by the timing of bacteriophage induced host cell lysis.

As shown in Table 1 there were differences in the infection parameters obtained for each infection temperature assayed. Both k and PFU/t obtained for cells grown and infected at 26 °C were greater and significantly different ($p < 0.05$) from those obtained at 4 and 37 °C. No increase in the phage titer was observed for cells grown and infected at 37 °C, thus no values regarding the latent-period, and PFU/t are presented. Additionally, the rate of cell lysis, evaluated by the amount of ATP released at 37 °C, was practically null. Accordingly, an increase in the amount of cells present in sus-
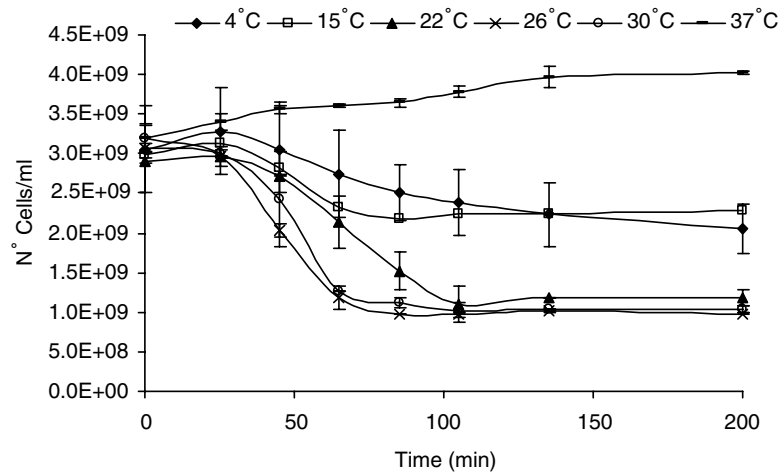

Fig. 1. Number of cells after phage ΦS1 infection of *P. fluorescens* cells grown and infected at different temperatures. Error bars correspond to standard deviations.
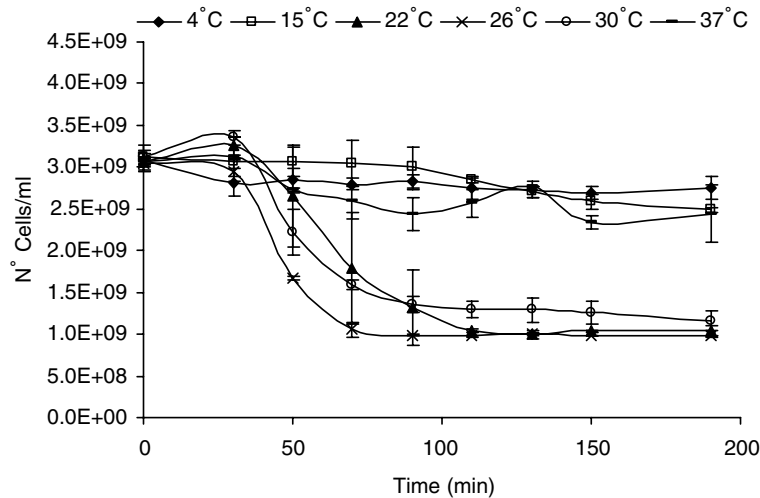
Fig. 2. Number of cells after phage ΦS1 infection of *P. fluorescens* cells grown at 26 °C and infected at different temperatures. Error bars correspond to standard deviations.
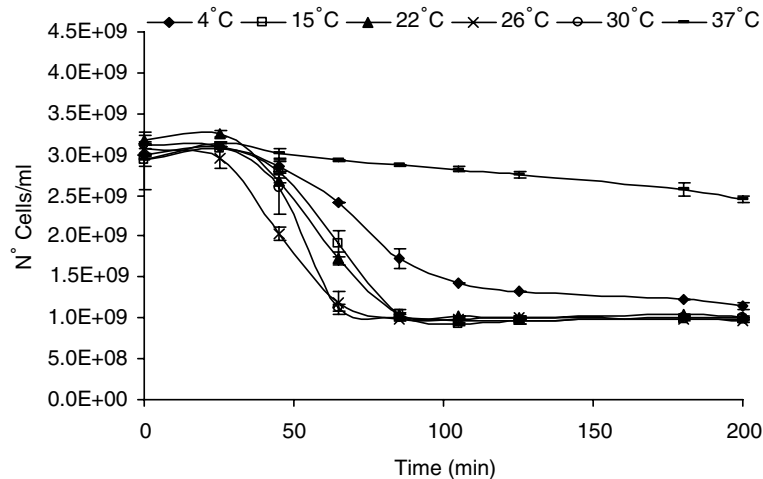

Fig. 3. Number of cells after phage ΦS1 infection of *P. fluorescens* cells grown at different temperatures and infected at 26 °C. Error bars correspond to standard deviations.

pension rather than a decrease was obtained under these conditions (Fig. 1).

From Table 1 it can also be observed that, in the case of cells grown at 26 °C the infection temperature of 26 °C resulted in significantly greater rates of cell lysis and PFU release ($p < 0.05$) compared to infection at 4 and 37 °C. Infection at 37 °C resulted in an increase in the latent-period comparing to phage infection at 26 °C.

Considering the effect of growth temperature, the results show that k and PFU/t obtained with cells grown at 4 and 37 °C were statistically different ($p < 0.05$) from those of cells grown at 26 °C. The infection parameters of cells grown at 37 °C were the lowest. At this temperature, no significant decrease in the number of cells present in suspension was observed (Fig. 3). The growth rates (μ, h^{-1}) obtained with the different growth temperatures had a considerable variation. The growth rate of *P. fluorescens*

was greater for 26 °C ($\mu = 0.534 \pm 0.101$ h^{-1}) than for 4 °C (0.355 ± 0.110 h^{-1}) and 37 °C (0.288 ± 0.158 h^{-1}).

In order to determine if there were different proteins expressed in cells grown at different temperatures, the outer membrane protein profiles of *P. fluorescens* were analyzed. Fig. 4 shows that a protein with a molecular weight of 17.5 ± 1 kDa was not present in cells grown at 4 and 37 °C. Furthermore, at 37 °C the protein with 99 ± 5 kDa was absent.

3.2. Effect of growth phase

The values of k and PFU/t (Table 2) after infection of cells at the exponential growth phase were statistically different ($p < 0.05$) from the ones obtained for stationary and decline phase cells. PFU/t obtained for cells at the exponential phase was approximately 2.3 times greater

Table 1
Rate of cell lysis (k), rate of phage release (PFU/t) and latent-period (L) (±standard deviations) obtained for *P. fluorescens* cells grown and infected at different temperatures

T_G (°C)	T_I (°C) 4			26			37		
	k (min^{-1})	PFU/t ($\times 10^{-8}$ PFU ml^{-1} min^{-1})	L (min)	k (min^{-1})	PFU/t ($\times 10^{-8}$ PFU ml^{-1} min^{-1})	L (min)	k (min^{-1})	PFU/t ($\times 10^{-8}$ PFU ml^{-1} min^{-1})	L (min)
4	0.010 ± 0.001	1.85 ± 1.09	75	0.014 ± 0.008	4.29 ± 0.07	50	n.d.	n.d.	
26	0.009 ± 0.003	3.22 ± 0.26	50	0.024 ± 0.008	16.90 ± 0.28	50	0.011 ± 0.002	0.12 ± 0.05[a]	75[a]
37	n.d.	n.d.		0.010 ± 0.001	0.35 ± 0.01	50	0.003 ± 0.001		

n.d., not determined.

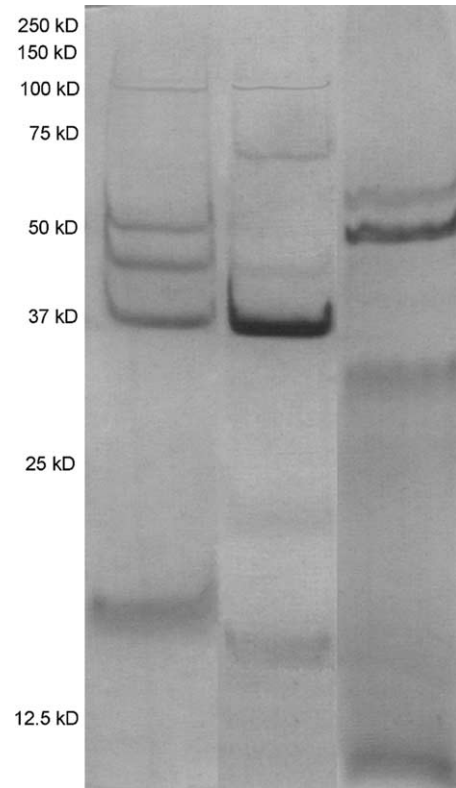[a] No increase in the titer was observed.

Fig. 4. Effect of growth temperature on the outer membrane proteins of *P. fluorescens*. SDS–PAGE (silver stained) of cells grown at 4 (lane 1), 26 (lane 2) and 37 °C (lane 3).

than after infection of cells at the stationary phase of growth and 8.6 times greater in the case of cells at the decline stage. Cells infected at this growth stage also exhibited an increase in the latent-period of phage infection.

In terms of the outer membrane proteins profiles, the proteins obtained at the different cell growth phases were the same. The only difference was in the concentration of the proteins separated in each band (Fig. 5).

3.3. Effect of growth and infection media

Table 3 shows that when cells grown in NBM were infected in GM the PFU/t presented was 3.9 times lower and significantly different ($p < 0.05$). When cells grown in GM were infected either in NBM or GM cell lysis and phage production were practically undetectable. The outer membrane protein profiles of cells grown in both media are shown in Fig. 6. The results indicate that the proteins with molecular weights of 17.5 ± 1 and 99 ± 5 kDa are not present in cells grown in GM.

4. Discussion

Most studies on phages have been performed under optimal conditions of phage infection and cell growth. However, conditions in the environment may not be

Table 2
Rate of cell lysis (k), rate of phage release (PFU/t) and latent-period (L) (±standard deviations) obtained for phage infection at 26 °C of *P. fluorescens* cells at different growth phases

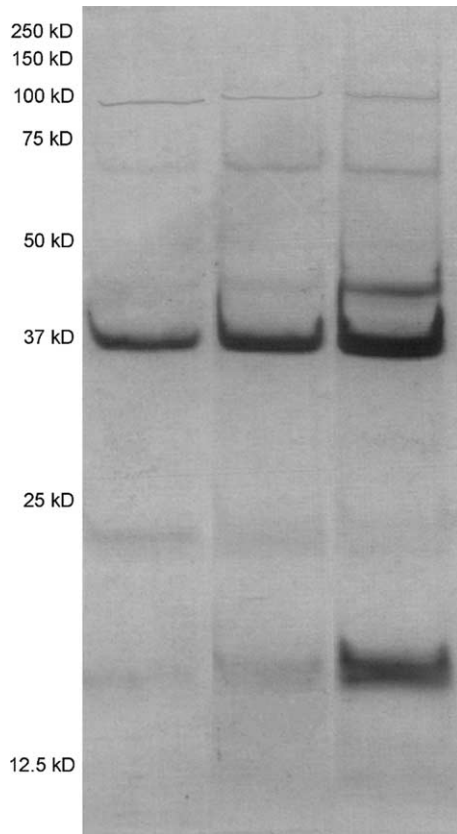| Growth phase	k (min^{-1})	PFU$_{MAX}$ (×10^{-10} PFU ml^{-1})	PFU/t (×10^{-8} PFU ml^{-1} min^{-1})	L (min)
Exponential	0.024 ± 0.008	8.50 ± 0.14	16.90 ± 0.28	50
Stationary	0.017 ± 0.002	8.15 ± 2.05	7.45 ± 0.18	50
Decline	0.012 ± 0.001	3.06 ± 0.57	1.96 ± 0.37	100

Fig. 5. Effect of growth phase on the outer membrane proteins of *P. fluorescens*. SDS–PAGE (silver stained) of cells at different growth phases: at the exponential phase (lane 1), at the stationary phase (lane 2) and at the decline phase (lane 3).

ideal. This study allows the understanding of the behaviour of phage ΦS1 facing non-ideal conditions, with special relevance to the effect of varying cell growth and infection temperatures, the growth phase of the cells and also the growth medium.

The results obtained showed that temperature had a great impact on phage efficacy (Fig. 1). In order to understand the effect of infection temperature and growth temperature on phage infection separately, it was fundamental to set a growth temperature as well as an infection temperature. 26 °C was the chosen temperature because it is the optimal temperatures of *P. fluorescens* growth and of phage infection (Fig. 1). Accordingly, cell growth and phage infection at 26 °C resulted in the lowest latent period and highest rates of cells lysis and phage release (Table 1).

Considering the effect of the infection temperature on phage performance, one possible reason for the decrease of phage activity at 37 °C when infecting cells grown at 26 °C is the inactivation of the phage when exposed to high temperatures [16]. To study the possibility of phage inactivation due to the effect of temperature and agitation, a solution containing phage and NBM media was incubated for 5 h, 130 rpm, and at the different temperatures assayed. During this period no loss of phage activity was observed; the titer remained constant (data not shown). Therefore, phage inactivation cannot explain this decrease in phage activity. It is well documented that the temperature influences the cell protein synthesising systems, usually high temperatures inhibit protein production. This might explain the low activity of phage when the infection temperature was 37 °C [17].

According to some authors, temperature can affect cell membrane permeability [18,19]. Maltouf and Labedan [20] suggested that a low temperature could prevent T5 phage infection of *Escherchia coli* by affecting the movement of the long DNA macromolecule itself or by rigidifying the host cell membranes. This phenomenon could be responsible for the decrease of phage activity when cells grown at 26 °C were submitted to infection at lower temperatures (Fig. 2). Phase transition of the outer membrane is very likely to occur when cells are grown at 26 °C and exposed to different temperatures. In the case of *Pseudomonas* species, membrane lipid phase transition can be observed over a wide temperature range, depending on the

Table 3
Rate of cell lysis (k), rate of phage release (PFU/t) and latent-period (L) (±standard deviations) obtained after phage infection of *P. fluorescens* cells grown in NBM and in 5 g/L glucose media

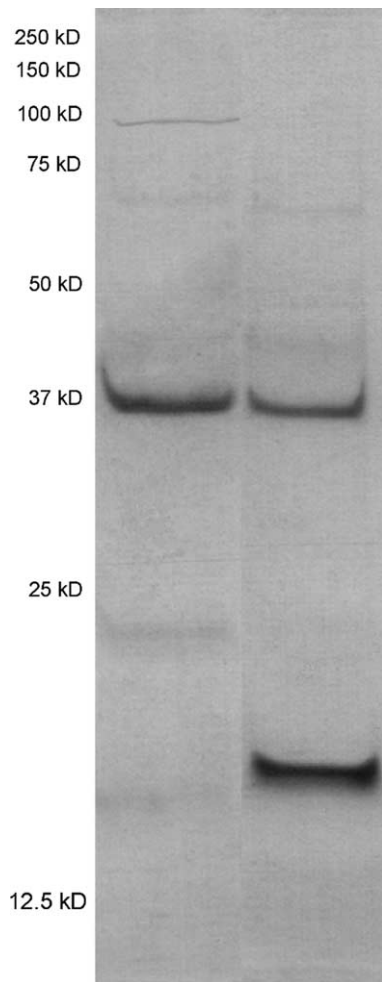| Growth media	Infection media			
	5 g/L glucose		NBM	
	k (min^{-1})	PFU/t (×10^{-8} PFU ml^{-1} min^{-1})	k (min^{-1})	PFU/t (×10^{-8} PFU ml^{-1} min^{-1})
5 g/L glucose	0.005 ± 0.001	0.29 ± 0.02	0.003 ± 0.001	0.087 ± 0.009
NBM	0.021 ± 0.001	4.28 ± 0.629	0.024 ± 0.008	16.90 ± 0.28

Fig. 6. Effect of growth media on the outer membrane proteins of *P. fluorescens*. SDS–PAGE (silver stained) of cells grown in NBM (lane 1), and grown in glucose media (lane 2). The numbers on the left indicate molecular weights.

growth media [18]. This phenomenon seems to have little influence on phage activity since the lytic profiles of cells infected at different temperatures (Fig. 2) are similar to those of cells grown and infected at the same temperatures in which membrane phase transition does not occur.

Considering the host growth temperature, the results obtained clearly demonstrated that the cell growth temperature influenced phage infection (Fig. 3). Cells grown at 26 °C yielded the highest amount of phage release per infected cell. This temperature was also the temperature at which the growth rate (μ) was the greatest. Other authors have reported that the rate of phage release increases with μ while the length of the latent period decreases [6]. Alterations of cell growth can occur simultaneously with modifications of macromolecular composition, metabolic rate and cell size [21,22]. It was suggested by Hadas et al. [6] that cell size rather than metabolic rate is one of the factors that affect T4 activity against *E. coli*. In the case of *P. fluorescens* the growth temperature influences cell size and shape [23].

Furthermore, at the optimal growth temperature the cells exhibit a greater concentration of proteins and RNA [24]. Therefore, in the present study, it is expected that cells grown at 26 °C are larger with a greater surface area for phage adsorption, which probably explains the higher phage efficiency. A key point of phage infection is the presence of cell wall receptors. The outer membrane of *P. fluorescens* grown at 37 °C did not exhibit the proteins of 17.5 ± 1 and 99 ± 5 kDa and cells grown at 4 °C did not exhibit a protein of 17.5 ± 1 kDa protein, both present in cells grown at 26 °C. These two proteins are probably essential for phage adsorption.

In the natural environment, cells exist mainly in the form of biofilm. Biofilms are characterised by cells at different growth stages embedded in an exopolysaccharide matrix. Consequently it was imperative to study the effect of cell growth stages in phage infection. The results showed that the infection of cells harvested at the exponential growth phase was more effective than that of cells at the stationary and decline stages as greater rates of cell lysis and number of phage release and the lowest latent-period were observed (Table 2). The transition from exponential growth to stationary state results in dramatic changes in cell morphology, rates of macromolecular synthesis and degradation, constitution and surface characteristics of the cell wall [25,16]. According to Hadas et al. [6], the latent and eclipse period, the burst size, and the rate of phage adsorption are influenced by host physiology (i.e., host quality). This fact may account for the increase of the latent-period when cells were at the decline stage. In terms of host quality, it is probable that this is inferior to that of cells at the exponential growth phase. Moreover, other authors have suggested that a delay of cell lysis appears to be linked to cell division, since infected stationary-phase cells released progeny virus only after resumption of cell growth [26,27]. This appears to be one of the explanations for the lower values of rate of cell lysis and rate of phage release when cells were at the stationary and decline stages. Cells harvested at the exponential, stationary and decline stages exhibited a similar cell wall protein composition (Fig. 5). Therefore, the decrease in phage efficiency when stationary and decline cells were infected was probably due to host quality rather than to the presence of cell wall receptors.

Phage infection can be influenced when cell growth conditions are unfavourable. Aspects of the medium composition, such as ionic strength [28,29] and pH [16,30,31] can greatly determine phage performance. The phage infection of *P. fluorescens* cells grown in glucose medium was inefficient (Table 3). One of the reasons for this was probably the absence of cell wall receptors in the outer membrane of cells grown in this medium (Fig. 6), as the 17.5 ± 1 and 99 ± 5 kDa proteins, identified as the probable cell wall receptors, were not expressed in these cells.

In any environment in which large numbers of bacteria are present and in which these cells are growing and eventually lysing it is probably possible to find a specific phage. But can the phages always be so efficient? According to this work and reports from other authors [1,32] phages are beyond doubt very important in the regulation of different species in the community of bacteria present in ecosystems. Under ideal conditions, such as temperature of growth and infection, as well as the presence of the essential nutrients, phages may act particularly well.

Acknowledgement

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