

Research article

Isolation and characterization of a T7-like lytic phage for *Pseudomonas fluorescens*

Sanna Sillankorva^{1,2}, Peter Neubauer² and Joana Azeredo*¹

Address: ¹IBB-Institute for Biotechnology and Bioengineering, Centre of Biological, Engineering, Universidade do Minho, Campus de Gualtar 4710-057, Braga, Portugal and ²Bioprocess Engineering Laboratory, Department of Process and Environmental Engineering and Biocenter Oulu, University of Oulu, P.O. Box 4300, FI-90014 Oulu, Finland

Email: Sanna Sillankorva - s.sillankorva@deb.uminho.pt; Peter Neubauer - peter.neubauer@oulu.fi; Joana Azeredo* - jazeredo@deb.uminho.pt

* Corresponding author

Published: 27 October 2008

Received: 16 April 2008

BMC Biotechnology 2008, 8:80 doi:10.1186/1472-6750-8-80

Accepted: 27 October 2008

This article is available from: <http://www.biomedcentral.com/1472-6750/8/80>

© 2008 Sillankorva et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (<http://creativecommons.org/licenses/by/2.0>), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Background: Despite the proven relevance of *Pseudomonas fluorescens* as a spoilage microorganism in milk, fresh meats and refrigerated food products and the recognized potential of bacteriophages as sanitation agents, so far no phages specific for *P. fluorescens* isolates from dairy industry have been closely characterized in view of their lytic efficiency. Here we describe the isolation and characterization of a lytic phage capable to infect a variety of *P. fluorescens* strains isolated from Portuguese and United States dairy industries.

Results: Several phages were isolated which showed a different host spectrum and efficiency of lysis. One of the phages, phage ϕ IBB-PF7A, was studied in detail due to its efficient lysis of a wide spectrum of *P. fluorescens* strains and ribotypes. Phage ϕ IBB-PF7A with a head diameter of about 63 nm and a tail size of about 13 × 8 nm belongs morphologically to the *Podoviridae* family and resembles a typical T7-like phage, as analyzed by transmission electron microscopy (TEM). The phage growth cycle with a detected latent period of 15 min, an eclipse period of 10 min, a burst size of 153 plaque forming units per infected cell, its genome size of approximately 42 kbp, and the size and N-terminal sequence of one of the protein bands, which gave similarity to the major capsid protein 10A, are consistent with this classification.

Conclusion: The isolated T7-like phage, phage ϕ IBB-PF7A, is fast and efficient in lysing different *P. fluorescens* strains and may be a good candidate to be used as a sanitation agent to control the prevalence of spoilage causing *P. fluorescens* strains in dairy and food related environments.

Background

Pseudomonas fluorescens is a Gram-negative psychrotrophic bacterium that can be divided into five biovars (I through V). This bacterium has frequently been isolated from milk and food related environments and characterized due to its relevant spoilage activity. *Pseudomonas* spp. are the main concern with regard to proteolytic degradation of milk, along with other less proteolytic degradative and

milk coagulant organisms such as *Achromobacter*, *Aeromonas*, *Flavobacterium*, and *Xanthomonas* spp. [1-5].

Studies with phages as control organisms of bacterial infections have increased in the recent years mainly due to the emergence of bacterial resistance to a vast number of antimicrobial agents. Phage therapy has again become a field worth of attention after years of abandonment in the

western world. Phage application to humans and to animals is already being performed, nevertheless there is hardly any literature on studies concerning industrial environments and the use of phages as sanitation agents. Although the spoilage ability of *P. fluorescens* is well known, there are no studies involving the use of phages capable of infecting dairy industry isolates of this host species. Essentially, the work on Pseudomonad phages is concentrated on *P. aeruginosa* phages due to the clinical relevance of this strain, which is an opportunistic pathogen affecting mainly immunocompromised people and those suffering from cystic fibroses. There is some work done with *P. fluorescens* and phages focused on co-evolution studies [6-8]. So far, bacteriophages for *P. fluorescens* were never closer characterized in view of their physico-chemical, morphological, and life cycle properties.

Here we describe the isolation of phages for the milk spoilage bacterium *P. fluorescens* and the further characterization of one phage with a very high lytic efficiency. The data of this work indicate that this effective phage belongs to the T7-group of bacteriophages and that it may be a good sanitizing agent for control of environments where *P. fluorescens* may provoke a quality risk.

Results

Isolation and host range characterization of promising lytic phages

The aim of this work was to isolate, select and characterize an effective lytic phage for a range of *P. fluorescens* strains. Therefore, initially several phages were isolated from raw sewage of a wastewater treatment plant that receives effluents from different dairy plants. Isolation was performed using the enrichment method with different test species of *P. fluorescens*, which had been earlier isolated from Portuguese and United States dairy plants. These isolates include different ribotypes and also strains with different enzymatic activities (see Table 1). Not all *P. fluorescens* are capable of producing degradative defects in processed milk. Therefore isolates from New York dairy industries which were able to produce extracellular enzymes that are problematic for milk products and cause their respective spoilage [3,9,10] were included in this study. All initial tests were performed with NY *P. fluorescens* isolates positive for protease, lipase, and lecithinase activity (Table 1), with exception of two of the strains, D3-197 and B1-020 which were able to produce only one type of enzyme, protease or lipase, respectively.

Altogether 17 phages were isolated and showed lytic activity against some of the different bacterial strains (Table 2). All the isolated phages were used in further screening assays using the phage spot test on bacterial lawns of each

Table 1: Characteristics of dairy *Pseudomonas* spp. used

Strain	Species identification	Origin	Ribotype	Prot/Le/Li	Source or reference
28	<i>P. fluorescens</i>	RSMT	*	+*/**	PT unpublished
33	<i>P. fluorescens</i>	RSMT	*	+*/**	PT unpublished
33B	<i>P. fluorescens</i>	RSMT	*	+*/**	PT unpublished
35	<i>P. fluorescens</i>	RSMT	*	+*/**	PT unpublished
37	<i>P. fluorescens</i>	RSMT	*	+*/**	PT unpublished
37B	<i>P. fluorescens</i>	RSMT	*	+*/**	PT unpublished
7	<i>P. fluorescens</i>	Teat cup shell	*	*/*/*	PT unpublished
8	<i>P. fluorescens</i>	Raw milk	*	*/*/*	PT unpublished
D3-149‡	<i>P. fluorescens</i>	Raw milk	409-S-3	+/+/+	[3]
D3-197‡	<i>P. fluorescens</i>	Processed milk	422-S-2	+/-/-	[3]
D3-331‡	<i>P. fluorescens</i>	Floor	57-S-8	+/+/+	[3]
D3-175‡	<i>P. fluorescens</i>	Processed milk	408-S-8	+/+/+	[3]
D1-045‡	<i>P. fluorescens</i>	Processed milk	50-S-8	+/+/+	[10]
B1-020‡	<i>P. fluorescens</i>	Potato isolate	57-S-5	-/-/+	[10]
D1-027‡	<i>P. fulva</i>	Raw milk	53-S-5	-/-/-	[10]
D2-160‡	<i>P. putida</i>	Raw milk	112-S-7	-/-/-	[10]
B1-041‡	<i>P. putida</i>	Raw milk	50-S-7	-/-/-	[10]
D1-026‡	<i>P. putida</i>	Raw milk	94-S-6	-/-/-	[10]
D1-046‡	<i>P. fragi</i>	Raw milk	72-S-3	-/-/-	[10]
B1-020‡	<i>P. fragi</i>	Raw milk	50-S-6	-/-/-	[10]

Isolate characteristics include origin, ribotypes and extracellular enzyme production [Protease (Prot), Lecithinase (Le) and Lipase (Li)]

RSMT – rubber short milk tube that connects the teatcup assemblies to the claw. * not performed, + positive for enzyme activity, - negative for enzyme activity.

‡ kindly provided by K. Boor (Milk Quality Improvement Program, Department of Food Science, Cornell University, Ithaca, N.Y.). All other bacterial strains were isolated from the dairy industry plant in Pacos de Ferreira, Portugal.

Table 2: Sensitivity of phages to different *P. fluorescens* isolates from dairy industry

Isolate	Phage ϕ IBB-																
	PF7A	PF7B	PF7C	PF7D	PF8	PF37B	PF33A	PF33B	PF33C	PF33D	D3-197A	D3-197B	D3-149A	D3-149B	D3-331A	D3-331B	D3-331C
7	C	C	C	C	C	C	-	-	-	-	C	C	T	T	-	-	-
8	C	C	C	C	C	C	C	-	-	C	C	C	C	C	-	-	-
28	C	C	C	C	C	C	C	-	-	-	T	T	C	C	-	-	-
33	C	C	C	C	C	-	C	T	T	T	T	-	-	C	T	-	-
33B	C	C	C	C	C	C	C	-	-	-	T	T	-	-	-	-	-
35	C	C	C	C	C	C	C	-	-	-	-	-	C	-	-	T	T
37	T	T	T	T	TT	-	-	-	-	-	-	-	-	-	-	-	-
37B	C	C	C	C	C	-	-	-	-	-	T	-	-	-	-	-	-
D3-149	C	C	C	C	C	C	-	-	-	-	C	C	C	C	-	-	-
D3-197	T	T	T	T	TT	C	-	-	-	-	C	C	-	-	-	-	-
D3-331	-	-	-	-	-	-	-	-	-	-	-	-	-	-	C	C	C
D3-175	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
B1-020	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
D1-045	C	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
D1-027	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
D2-160	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
B1-041	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
D2-026	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
D1-046	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
B1-020	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Group			I			2	3	4	5	6	7	8	9	10		11	

* not tested, C – clear phage plaque, T – turbid phage plaque, TT – very turbid, – no phage plaque

different strains in order is to characterize their host range and to select the phage capable of infecting the greatest number of *P. fluorescens* strains. This screening procedure is the basis of phage typing methods and it enabled the differentiation of 14 different *P. fluorescens* strains as they resulted in dissimilar phage screenings profiles.

According to the phage lytic profiles, the phages were divided into 11 different groups based on the host susceptibility, as indicated on the bottom of Table 2. Each group is characterized by the same lytic profile of the phages for the same host strains, which either were sensitive to the distinct phages, by showing clear or turbid plaques, or resistant (no phage plaques observed). From these results it seems very likely that the phages belonging to different groups also represent different phages. Several phages were able to lyse most of the *P. fluorescens* isolates from the Portuguese dairy plant, however some phages were more host-specific, such as the phages isolated for *P. fluorescens* strains D3-331 and 33. Phages ϕ IBB-D3-331A to C only formed clear plaques on the *P. fluorescens* isolate D3-331 and a turbid plaque in one more isolate. Phages ϕ IBB-33B to D caused turbid plaques on the host which was used for isolation and only ϕ IBB-33D was additionally able to produce clear plaques on host strain 8. The phages with the widest host range belonged to group 1 and where isolated with the *P. fluorescens* strains number 7 and 8 respectively. From the phage group 1, phage ϕ IBB-PF7A had the largest plaque diameter (data not shown). Therefore, phage ϕ IBB-PF7A was selected for further characterization studies and tested for lysis ability in other characterized *P. fluorescens* isolates and other *Pseudomonas* species provided (*P. putida*, *P. fragi* and *P. fulva*) by the Cornell University (NY) which were unable to produce extracellular enzymes. Phage ϕ IBB-PF7A was only able to form plaques on *P. fluorescens* strains and did not lyse the three *Pseudomonas* species tested (Table 1).

P. fluorescens ribotypes tested where: 409-S-3, 422-S-2, 408-S-8, 50-S-8, and 57-S-5. Ribotypes 50-S-8 and 57-S-5 belong to *P. fluorescens* biovar II and the respective isolates, B1-020 and D1-045, cluster between the *P. fluorescens* and *P. putida* lineages in the so-called B3 cluster. Ribotypes 409-S-3, 408-S-8 and 422-S-2 have not been classified according to the biovar to which they belong. According to Dogan and Boor [3], from 42 *Pseudomonas* spp. isolated from four New York dairy plants, 21 isolates had *P. fluorescens* ribotypes. Furthermore, only 19% of the total *Pseudomonas* spp., or approximately 8 *P. fluorescens* ribotypes, are found in more than one dairy plant, while the other 81% are dairy plant specific ribotypes. Taking this into account and the fact that phage ϕ IBB-PF7A could infect 13 different strains of which 5 were had different ribotypes suggests that ϕ IBB-PF7A may be an interesting

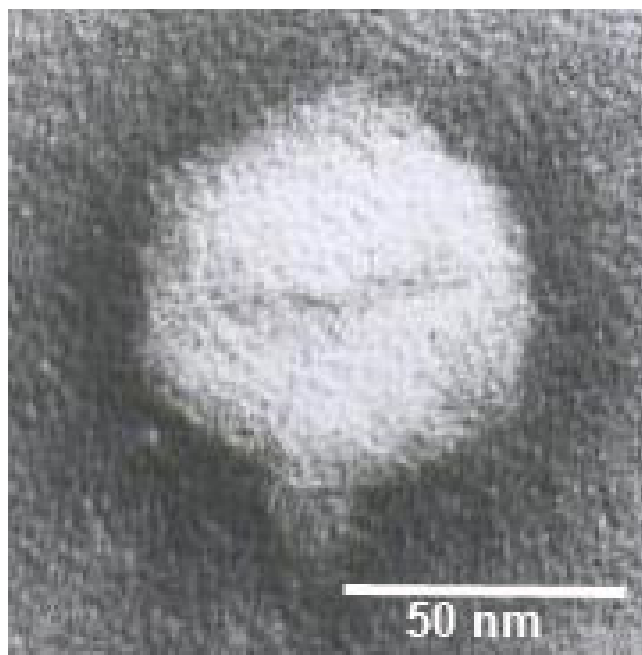


Figure 1
Transmission electron micrographs of *P. fluorescens* phage ϕ IBB-PF7A.

candidate for sanitizing applications and therefore was further characterized.

Morphology of the phage particles

Morphological characterization of phages using Transmission Electron Microscopy (TEM), one of the most used methods to classify phages, showed that phage ϕ IBB-PF7A has a pentagonal outline indicating an icosahedral nature (Fig. 1). According to the TEM micrograph, phage ϕ IBB-PF7A belongs to the *Podoviridae* family, which is characterized by phages with a short non-contractile tail. Phage ϕ IBB-PF7A has a very short tail most likely belonging to type C in Bradley's classification [11]. Furthermore, the diameters of phage ϕ IBB-PF7A's icosahedral capsid and tail length (Table 3) are similar to typical morphological values observed in members of the T7 phage group (H.-W. Ackermann, personal communication).

Phage DNA studies

The isolation of phage DNA and the application of restriction enzymes allow the approximate determination of the genomic size of phages and evaluate if the size is consistent with the T7 classification proposed through the TEM observation. The restriction of phage ϕ IBB-PF7A DNA was performed with EcoRI and HindIII. The sum of the fragments resulted in the following genomic sizes: 41,945 bp and 41,870 bp with EcoRI and HindIII, respectively (Fig. 2). The approximate genomic size of ϕ IBB-PF7A with

Table 3: Phage ϕ IBB-PF7A plaque features, life cycle parameters and morphological characteristics determined from TEM micrographs

Plaque		Morphology*	Life Cycle
Diameter: 4–6 mm	Head size: 63 nm	Tail size: 13 nm × 8 nm, tapering	Latent period: 15 min
Halo size: 0.5 mm			Adsorption rate: 5.58×10^{10} mlmin ⁻¹
			Eclipse period: 10 min
			Rise period: 25 min
			Burst size: 153 PFU per infected cell

* Determinations performed by Dr. H. W. Ackermann, Laval University, Quebec, Canada.

EcoRI and HindIII is approximately 42 kbp which is in the range of T7 phages that varies between 38,208 bp and 45,874 bp as further discussed below.

Analysis of phage structural proteins

To further characterize ϕ IBB-PF7A, its protein composition was analyzed by SDS-PAGE and 3 bands were N-terminally sequenced. SDS-PAGE allows the observation of the number of structural proteins present on this phage

and to evaluate the similarities with protein profiles of known T7 structural proteins.

At least 16 bands can be clearly distinguished in the gradient gel (Fig. 3 and Table 4) ranging from approximately 16 to 140 kDa. The most predominant polypeptide band appeared at a size of approximately 45 kDa. This band (p3) could be assigned to the T7 major capsid protein 10A by its size and by its N-terminal sequence determination of the first 10 amino acid residues. This protein has 80% of sequence homology with ϕ YeO3-12 and T3 phages, which are both members of the T7 phage supergroup. Also four other protein bands could be correlated with T7 structural proteins: T7 tail fiber protein in the monomeric form (p1 in Fig. 3); head-tail connector protein (p2), cap-

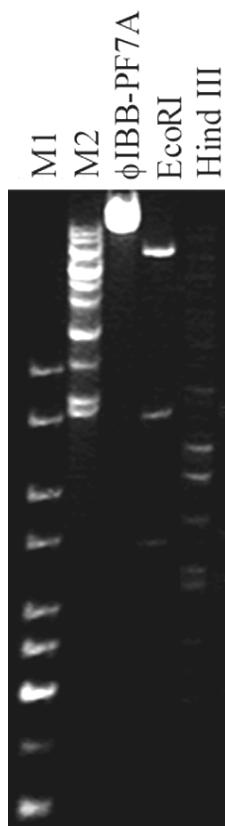


Figure 2
Restriction analysis of phage ϕ IBB-PF7A DNA. Undigested phage DNA and phage DNA digested with EcoRI and HindIII. Lanes M1 and M2: 1 kb and lambda mix marker DNA ladders.

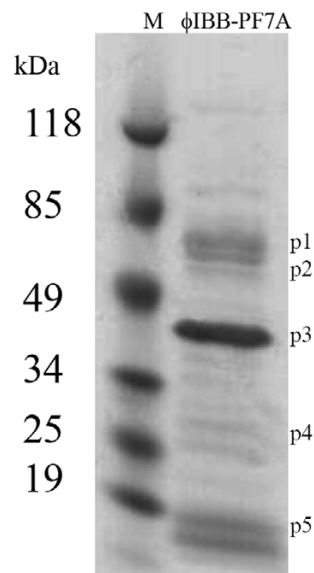


Figure 3
SDS-polyacrylamide gel electrophoresis analysis of phage ϕ IBB-PF7A structural proteins. Phage lysate was mixed with Laemmli buffer containing SDS, boiled for 10 min, and loaded on a 4–20% gradient gel that was electrophoresed with Tris-glycine running buffer. Lane M: molecular weight marker. p1 to p5 mark sizes of typical T7 phage structural proteins. Further explanations in the Results section.

Table 4: Phage ϕ IBB-PF7A structural proteins

MW (kDa) ^a	Probable T7 protein	aa sequence	Identity (%)	BLASTp similarity (phage and accession nr.)	Alignment
137.3					
98.4					
85.6					
74.5					
68.5					
63.1 (p1)	Tail fiber (monomeric form of gp17) ¹	KEVLFGDS	80	Hyp. protein (phage BA3, YP001552271)	KEVLFG – DS KEVLFG – DS KEVLFLGND
59.7 (p2)	Head-tail connector (gp8) ¹				
50.5	-	No sequence	-	N-terminally blocked	-
45.2 (p3)	Major capsid (gp10A) ²	AQMGGGQQIG	80	Major capsid protein 10A (phage ϕ YeO3-12, NP_052109; phage T3, NP_523335)	AQMGGGQQIG A – QGGGQQIG ANIQQGGQQIG
39.3					
38.2					
30.6 (p4)	Capsid assembly (gp9) ¹				
29.0					
25.9					
18.6 (p5)	Internal virion B (gp14) ¹				
16.6					

Molecular weights of phage ϕ IBB-PF7A structural proteins were determined from SDS-PAGE gels and compared with known T7 structural proteins with approximate weights, N-terminal sequence of proteins, similarities and respective accession numbers

^a) Average from 2 separate determinations; ¹ Kovalyova and Kropinski, 2003; ² Pajunen et al. 2000.

sid assembly protein (p4), and also the internal virion protein B (p5) [12,13]. One of these four bands, the 63.1 kDa band (p1), gave a signal in the N-terminal sequence determination. The 63.1 kDa band resulted in 8 clearly determined amino acid residues and was 80% similar to a hypothetical protein of *Thalassomonas* phage BA3. Like ϕ IBB-PF7A, phage BA3 also belongs to the *Podoviridae* family of phages but has not yet been further classified. The N-terminal sequence of the 50.5 kDa protein band did not give sequence which indicates that this protein is N terminally blocked. The analysis of ϕ IBB-PF7A structural proteins clearly demonstrate that there are comparable proteins to other T7-like phages and like other phages belonging to this group, the most predominant structural

protein is the major capsid protein 10A as verified by N-terminal sequencing.

Phage growth characteristics

The EM, genomic and structural protein studies permit to classify ϕ IBB-PF7A as a T7-like phage. Furthermore, it was our aim to characterize ϕ IBB-PF7A's life cycle and adsorption ability. Firstly, one-step growth studies were performed to identify the different phases of a phage infection process. After infection of ϕ IBB-PF7A's host, the *P. fluorescens* isolate nr. 7, the phage growth cycle parameters – the latent period, eclipse period, rise period, and burst size, were determined from the dynamical change of the number of free and total phages (Fig. 4 and Table 3).

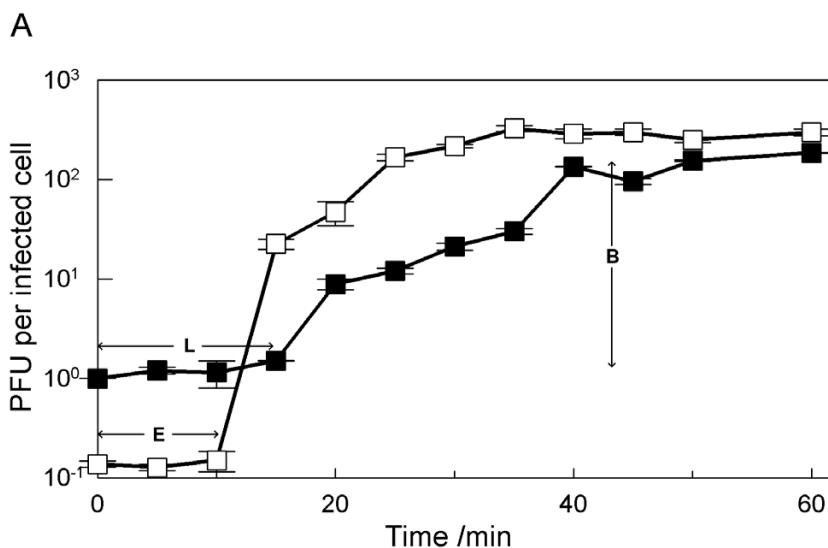


Figure 4
One-step growth curve of phage ϕ IBB-PF7A in *P. fluorescens* at RT. Shown are the PFU per infected cell in untreated cultures (■) and in chloroform-treated cultures (□). The phage growth parameters are indicated in the figure and correspond to: E-eclipse period; L-latent period and B-burst size.

In the system studied, the eclipse and latent periods of ϕ IBB-PF7A were very short, 10 and 15 min, respectively. ϕ IBB-PF7A yielded a burst size of 153 PFU per infected cell after 25 min at RT. These phage life cycle values are in conformity with the values normally observed for T7 group phages.

The adsorption efficiency of phages to the host was estimated with cells in the early logarithmic growth phase. The experiment was carried out at room temperature under constant shaking (150 rpm) and a phage inoculum of MOI = 0.01. The number of free phages was calculated from the PFU of chloroform-treated samples within 15 min after inoculation (Fig. 5). Phage ϕ IBB-PF7A appears to have two adsorption phases: a very rapid adsorption to its host during the first 5 min is followed by a slower rate of attachment after 5 min. The number of free phages was below 10% already within 5 min and below 5% within 15 min after infection (Fig. 5). The adsorption rate, which represents the phage adsorption affinity towards the host, was calculated according to Barry and Walter [14] for a period of 5 min. The adsorption rate constant of phage ϕ IBB-PF7A was calculated to be $5.58 \times 10^{-10} \text{ ml min}^{-1}$ (Table 3) which is similar to other T7 rates in literature [14].

Discussion

Pseudomonas fluorescens is a major milk product contaminant as well as spoilage causing agent of fresh poultry and refrigerated foods, in particular of refrigerated meats

[1,3,15-18]. Product contamination occurs at different stages of processing which shows that it is difficult to maintain the processing environment clean from this bacterium mainly due to the ecologic diversity and multiple origins of *Pseudomonas* spp. [3]. Phages have been suggested as alternative anti-microbial agents for a variety of pathogenic bacteria found in food processing environments [19-23]. So far, no phages have been closely studied for their ability to infect the spoilage causing bacterium *P. fluorescens*. The bacterial isolates tested in this work

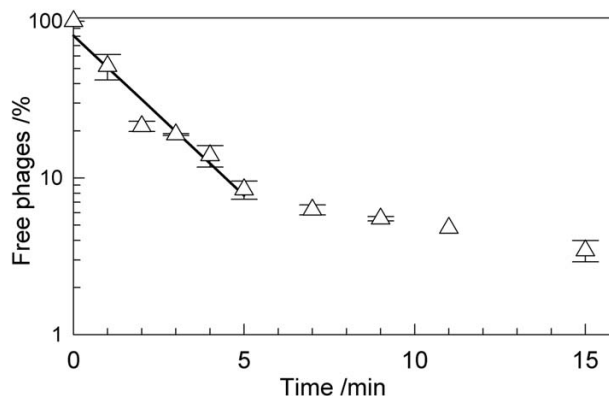


Figure 5
Percentage of free ϕ IBB-PF7A phages after infection of steady-state *P. fluorescens* at a MOI of 0.01.

include different ribotypes and isolates capable of producing extracellular enzymes, such as protease, lecithinase and lipase that cause spoilage of milk products. A number of phages were isolated for *P. fluorescens*, some of which exhibited a high efficiency in lysing different *P. fluorescens* isolates while others had a narrower host range. This shows that a variety of lytic *P. fluorescens* phages can be easily isolated from the environment and their use as alternative sanitation agents will most likely be feasible for application in food and dairy industry as demonstrated by studies with *P. fluorescens* biofilms subjected to phage [24].

Of all the phages isolated, phage ϕ IBB-PF7A was selected for further characterization. This novel highly lytic phage ϕ IBB-PF7A has a strong virulence towards a number of *P. fluorescens* present on Portuguese and American dairy facilities. The verification of the ability of phage ϕ IBB-PF7A in infecting isolates capable of producing extracellular enzymes isolates was an exceptionally important result and is an additional argument for phage based sanitation – it is possible to select phages only for a specific range of bacteria, in this case for the enzyme producing bacteria that are the major spoilage causative microorganisms.

The structural proteins are similar with known T7 proteins. Five distinct protein bands were identified according to their size representing the T7 tail fiber protein, the minor capsid protein, the major capsid protein 10A, the capsid assembly protein, and the internal virion protein B [12,13]. In fact, N-terminal sequencing identified one of the protein bands as the T7-like major capsid protein 10A.

Phage ϕ IBB-PF7A has a genome size of about 42 kbp (Fig. 5) which is very close to the T7 phage ϕ KMV (42,519 bp), a *Pseudomonas aeruginosa* phage [25]. This is well in the range of genome sizes of other T7-like phages; the smallest T7-like phage genome sequenced so far is the *E. coli* phage T3 having a size of 38,208 bp and the largest T7-like phage, a *Vibrio parahaemolyticus* phage, has a size of 45,874 bp [25].

The adsorption rate of 5.58×10^{-10} ml min⁻¹ obtained through the one-step growth curve (Fig. 2) is also well in accordance with other T7-like phages, with values varying between 4.5×10^{-10} and 8.9×10^{-10} ml min⁻¹, depending on whether the bacteria are alive or were killed [14].

The use of phages of the *Podoviridae* group, specifically T7-like phages, as therapeutic agents has been reported for different bacteria. *P. aeruginosa* T7-like phages ϕ KMV, LKD16, and LKA1 have been suggested as good biological agents due to their ability to infect a variety of clinical *P. aeruginosa* isolates [25,26]. Phage PppW-4 for *P. plecoglossida* has been suggested equally suggested for combating

the hemorrhagic ascites disease in cultured ayu fish [27,28]. Aside from the high rate of development of T7-like phages if e.g. compared to T4 phages that are being used in animal therapy, T7-like phages are also interesting for their small genome, which decreases the chance of transferring extended fragments of chromosomal DNA with pathogenicity islands [29] to host strains.

The data obtained in this study support the hypothesis that phage ϕ IBB-PF7A can be a good candidate for phage based sanitation in food processing environments, preventing product spoilage due to extracellular enzyme producing *P. fluorescens* strains.

Conclusion

To our knowledge, this is the first report with a detailed study of phages isolated for dairy *P. fluorescens* isolates. The newly isolated T7-like phage has an increased potential for lysing a variety of isolates and has several attractive features such as very short replication times and very fast adsorption ability which makes the phage a promising candidate for therapeutic or sanitation purposes.

Methods

Bacteria and growth conditions

The bacterial strains (see Table 1) were previously isolated from Portuguese and United States dairy plants. All bacteria were grown at 30 °C in Tryptic Soy Broth (TSB, Fluka). Solid TSA medium contained 1.2% w/v of Bacto™ agar (Difco) and the soft agar top-layer contained 0.6% of Bacto™ agar. All bacteria were subcultured once and glycerol stocks were done and stored frozen at -80 °C until further use.

Bacteriophage isolation

Bacteriophages were isolated from a sewage treatment plant (ETAR de Esposende, Portugal). This wastewater treatment plant was selected because it receives effluents from many dairy industries and cow farms. For selection of phages 200 ml of raw sewage sample was added to a 1 L Erlenmeyer flask containing 200 ml of 2 × TSB medium and 50 μ l of the respective bacterial test species with an optical density of 1.0. The solution was incubated for 24 h at 150 rpm and 30 °C on a rotary shaker and was afterwards centrifuged (10 min, 10,000 × g, 4 °C). The clear supernatant was chloroform treated and phage detection was done by spotting the phage lysate on bacterial lawns. These plates were incubated at 30 °C for 12 h and inspected for plaques. Plaques were tested for containing only one single type of phage by repeated transfer of the lysates to new plates. Final lysates were stored at 4 °C for further use.

Host range of phages and selection of the most efficient phage

The isolated phages were investigated for host range specificity and lysis efficiency (no lysis, clear plaque, and turbid plaque) in screening tests against different *P. fluorescens*. Bacterial lawns of different *P. fluorescens* were propagated on TSA plates and 10 µl droplets of phages (1×10^4 up to 1×10^7 PFU ml⁻¹) were put on the lawns. The plates were incubated 18 h and checked for presence of plaque. The most efficient phage, the one which was able to lyse the greatest number of hosts, was selected for further studies. The selection criteria included the lysis profiles, plaque clarity and size. Phages were named according to the bacteria from which they were isolated [e.g. phage φIBB-PF7 stands for Institute for Biotechnology and Bioengineering (IBB), the host bacterium *Pseudomonas fluorescens* (PF) and sample number 7]. In case different plaque sizes were obtained using the same host bacterium for phage isolation, the plaques were further purified and considered different phages and a caps letter was then added at the end of the phage name, i.e. phage φIBB-PF7A.

Bacteriophage propagation and concentration

Small-scale concentration of phages was performed by spreading phages on the top-agar layer containing the respective host bacterium by plaque picking and using sterile paper strips. Briefly, 10 µl of isolated phage lysate was added onto a TSA Petri dish containing a soft top-agar layer with bacteria and spread with the help of sterile paper strips. After incubation the lysate from a clear Petri dish was eluted with SM buffer (5.8 g l⁻¹ NaCl, 2 g l⁻¹ MgSO₄ × 7 H₂O, 50 ml 1 M Tris, pH 7.5) and further purified with chloroform and stored at 4 °C. These solutions were then used for preparing concentrated phage solutions in larger scale using the plate lysis and elution method as described by Sambrook & Russel [30] with some modifications. Briefly, a top agar was prepared containing 1 ml of phage solution and 1 ml of a bacterial overnight culture in 30 ml of soft-agar. This agar was added to 250 ml T-flasks with a thin bottom layer of TSA. After solidification of the top agar layer the T-flasks were incubated at 30 °C overnight. Afterwards, the flasks were eluted with SM buffer and the phage lysate was first concentrated with PEG 8000 and then purified with chloroform. Samples in SM buffer were stored at 4 °C until further use.

Phage titration

Bacteriophage titer was analysed as described by Adams [31]. Briefly, 100 µl of diluted phage solution, 100 µl of a bacterial overnight culture, and 3 ml of molten agar were mixed in a glass tube and poured into a TSA containing Petri dish. Plates were incubated for 18 h after which plaque forming units (PFU) were counted.

Electron microscopy

Bacteriophage particles were sedimented at 25,000 × g for 60 min using a Beckman (Palo Alto, CA) J2-21 centrifuge with a JA 18.1 fixed-angle rotor. Bacteriophages were washed twice in 0.1 M ammonium acetate (pH 7.0), deposited on copper grids provided with carbon-coated Formvar films, stained with 2% potassium phosphotungstate (PT, pH 7.2), and examined in a Philips EM 300 electron microscope (performed by Dr. H. W. Ackermann, Laval University, Quebec, Canada).

SDS-PAGE

Purified phage solution was precipitated with 4 volumes of ice-cold acetone. After centrifugation (1,600 × g, 20 min, 4 °C) the pellet was air-dried and resuspended in PBS buffer (8 g l⁻¹ NaCl, 0.2 g l⁻¹ KCl, 0.2 g l⁻¹ KH₂PO₄, 1.44 g l⁻¹ Na₂HPO₄ × 2H₂O, pH 7.5). SDS-PAGE was carried out according to Laemmli [32]. Briefly, 24 µl of sample were added to 8 µl of 4 × Laemmli buffer and boiled for 10 min. Samples were then loaded to 4 – 20% PAGEr precast gels (Cambrex) and electrophoresed with Tris-glycine buffer. After electrophoresis the gels were stained with Bio-safe Coomassie (BioRad).

DNA isolation and restriction enzyme digestion

Phage DNA was isolated using a Wizard Lambda Preps DNA purification system (Promega, Madison, Wis.) according to the manufacturer's instructions.

Phage DNA was digested with the following restriction enzymes: Eco RI, Eco RV, Hind III, and Tsp 509I according to the instructions provided by the manufacturer. All restriction enzyme digestions were performed in triplicate. Electrophoresis at 15 V for 24 h was performed with a 0.7% TAE agarose gel stained with ethidium bromide. A 1 kb DNA ladder and Lambda Mix Marker (both from Fermentas) were used as molecular weight markers to calculate the sizes of the phage DNA fragments.

N-terminal amino acid sequencing of proteins

Phage proteins separated in 4 – 20% gradient gels were transferred to PVDF membranes (500V, 1.25 h) and stained with Bio-safe Coomassie brilliant blue solution (Bio-Rad) (1 min) and de-stained with 50% methanol. The membrane was rinsed with milliQ water for 5 min and let dry. The chosen bands were excised from the membrane and subjected to Edman chemistry for determining the N-terminal sequences. The sequencing was performed on a Procise™ 492 protein sequencer (PE Applied Biosystems).

One-step growth curve

One-step growth curves were performed as described by Pajunen *et al.* [13] with some modifications. Briefly, 10 ml of a mid-exponential-phase culture were harvested by

centrifugation (7,000 × g, 5 min, 4°C) and resuspended in 5 ml fresh TSB medium in order to obtain an OD₆₀₀ of 1.0. To this suspension, 5 ml of phage solution were added in order to have a MOI of 0.001 and phages were allowed to adsorb for 5 min at room temperature. The mixture was then centrifuged as described above and the pellet was resuspended in 10 ml of fresh TSB medium. Two samples were taken every 5 min over a period of 1 h. The first sample was plated immediately without any treatment and the second set of samples was plated after treatment with 1% (vol/vol) chloroform to release intracellular phages.

Bacteriophage adsorption

Bacteria in the steady-state growth phase were diluted in TSB to an optical density (OD₆₀₀) of 1.0. Afterwards, 10 ml of the bacterial suspension and 10 ml of phage solution were mixed in order to give a multiplicity of infection (MOI) of 0.01. The mixture was incubated at room temperature with shaking (150 rpm, Rotamax 120, Heidolph) and samples were collected every minute during a total period of 15 min. Samples were immediately chloroform-treated, diluted and plated on TSA plates. After overnight incubation at 30°C phage plaques were counted.

Authors' contributions

SS performed all practical experimental work and wrote the manuscript. PN contributed with his experiences in phage cultivation and JA with her competence in biofilm control. JA and PN supervised the work. The final manuscript was read and accepted by all coauthors.

Acknowledgements

The authors thank Dr. Maria João Vieira (University of Minho, Braga, Portugal) and Dr. K. Boor (Cornell University, N.Y., U.S.A.) for generously providing the bacteria for this study, M.Sc. Zélia Fernandes for providing sewage samples from the wastewater treatment plant of Esposende and the help provided by Ulf Liebal and Mirja Krause in the adsorption experiments. The authors are also grateful to Dr. Hans-W. Ackermann (Laval University, Quebec, Canada) for the TEM analysis of the phages. This work was supported by a grant (SFRH/BD/18485/2004) from the Portuguese Foundation for Science and Technology (FCT).

References

1. Arnaut-Rollier I, De Zutter L, Van Hoof J: **Identities of the *Pseudomonas* spp. in flora from chilled chicken.** *International Journal of Food Microbiology* 1999, **48**:87-96.
2. Bodilis J, Calbrix R, Guerillon J, Merieau A, Pawlak B, Orange N, Bar-ray S: **Phylogenetic relationships between environmental and clinical isolates of *Pseudomonas fluorescens* and related species deduced from 16S rRNA gene and OprF protein sequences.** *Systematic and Applied Microbiology* 2004, **27**:93-108.
3. Dogan B, Boor KJ: **Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants.** *Applied and Environmental Microbiology* 2003, **69**:130-138.
4. Peneau S, Chassaing D, Carpentier B: **First evidence of division and accumulation of viable but nonculturable *Pseudomonas fluorescens* cells on surfaces subjected to conditions encountered at meat processing premises.** *Applied and Environmental Microbiology* 2007, **73**:2839-2846.
5. Wang L, Jayarao BM: **Phenotypic and genotypic characterization of *Pseudomonas fluorescens* isolated from bulk tank milk.** *Journal of Dairy Science* 2001, **84**:1421-1429.
6. Morgan AD, Brockhurst MA, Lopez-Pascua LDC, Pal C, Buckling A: **Differential impact of simultaneous migration on coevolving hosts and parasites.** *Bmc Evolutionary Biology* 2007, **7**.
7. Buckling A, Wei Y, Massey RC, Brockhurst MA, Hochberg ME: **Antagonistic coevolution with parasites increases the cost of host deleterious mutations.** *Proceedings of the Royal Society B-Biological Sciences* 2006, **273**:45-49.
8. Brockhurst MA, Buckling A, Rainey PB: **Spatial heterogeneity and the stability of host-parasite coexistence.** *Journal of Evolutionary Biology* 2006, **19**:374-379.
9. Craven HM, Macauley BJ: **Microorganisms in Pasteurized Milk After Refrigerated Storage. I. Identification of Types.** *Australian Journal of Dairy Technology* 1992, **47**:38-45.
10. Wiedmann M, Weilmeyer D, Dineen SS, Ralyea R, Boor KJ: **Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk.** *Applied and Environmental Microbiology* 2000, **66**:2085-2095.
11. Bradley DE: **Ultrastructure of Bacteriophages and Bacteriociens.** *Bacteriological Reviews* 1967, **31**:230.
12. Kovalyova IV, Kropinski AM: **The complete genomic sequence of lytic bacteriophage gh-1 infecting *Pseudomonas putida* – evidence for close relationship to the T7 group.** *Virology* 2003, **311**:305-315.
13. Pajunen M, Kiljunen S, Skurnik M: **Bacteriophage phi YeO3-12, specific for *Yersinia enterocolitica* serotype O : 3, is related to coliphages T3 and T7.** *Journal of Bacteriology* 2000, **182**:5114-5120.
14. Barry GT, Goebel WF: **The Effect of Chemical and Physical Agents on the Phage Receptor of Phase-li *Shigella sonnei*.** *Journal of Experimental Medicine* 1951, **94**:387-400.
15. Arnaut-Rollier I, Vauterin L, De Vos P, Massart DL, Devriese LA, De Zutter L, Van Hoof J: **A numerical taxonomic study of the *Pseudomonas* flora isolated from poultry meat.** *Journal of Applied Microbiology* 1999, **87**:15-28.
16. Cousin MA: **Presence and Activity of Psychrotrophic Microorganisms in Milk and Dairy-Products – A Review.** *Journal of Food Protection* 1982, **45**:172-207.
17. Cromie S: **Psychrotrophs and Their Enzyme Residues in Cheese Milk.** *Australian Journal of Dairy Technology* 1992, **47**:96-100.
18. Sorhaug T, Stepaniak L: **Psychrotrophs and their enzymes in milk and dairy products: Quality aspects.** *Trends in Food Science & Technology* 1997, **8**:35-41.
19. Huff WE, Huff GR, Rath NC, Donoghue AM: **Evaluation of the influence of bacteriophage titer on the treatment of colibacillosis in broiler chickens.** *Poult Sci* 2006, **85**:1373-1377.
20. Atterbury RJ, Van Bergen MA, Ortiz F, Lovell MA, Harris JA, De Boer A, Wagenaar JA, Allen VM, Barrow PA: **Bacteriophage therapy to reduce salmonella colonization of broiler chickens.** *Appl Environ Microbiol* 2007, **73**:4543-4549.
21. Barrow PA: **Novel approaches to control of bacterial infections in animals.** *Acta Vet Hung* 1997, **45**:317-329.
22. Higgins JP, Higgins SE, Guenther KL, Huff W, Donoghue AM, Donoghue DJ, Hargis BM: **Use of a specific bacteriophage treatment to reduce *Salmonella* in poultry products.** *Poult Sci* 2005, **84**:1141-1145.
23. Loc Carrillo C, Atterbury RJ, el-Shibiny A, Connerton PL, Dillon E, Scott A, Connerton IF: **Bacteriophage therapy to reduce *Campylobacter jejuni* colonization of broiler chickens.** *Appl Environ Microbiol* 2005, **71**:6554-6563.
24. Sillankorva S, Neubauer P, Azeredo J: ***Pseudomonas fluorescens* biofilms subjected to phage phiBB-PF7A.** *BMC Biotechnology* 2008, **8**:79.
25. Lavigne R, Burkal'tseva MV, Robben J, Sykilinda NN, Kurochkina LP, Grymonprez B, Jonckx B, Krylov VN, Mesyanzhinov VV, Volckaert G: **The genome of bacteriophage phi KMV, a T7-like virus infecting *Pseudomonas aeruginosa*.** *Virology* 2003, **312**:49-59.
26. Ceysens PJ, Lavigne R, Mattheus W, Chibeu A, Hertveldt K, Mast J, Robben J, Volckaert G: **Genomic analysis of *Pseudomonas aeruginosa* phages LKD16 and LKA1: Establishment of the phiKMV subgroup within the T7 supergroup.** *Journal of Bacteriology* 2006, **188**:6924-6931.
27. Park SC, Shimamura I, Fukunaga M, Mori KI, Nakai T: **Isolation of bacteriophages specific to a fish pathogen, *Pseudomonas***

- plecoglossida, as a candidate for disease control.** *Applied and Environmental Microbiology* 2000, **66**:1416-1422.
28. Park SC, Nakai T: **Bacteriophage control of *Pseudomonas plecoglossida* infection in ayu *Plecoglossus altivelis*.** *Diseases of Aquatic Organisms* 2003, **53**:33-39.
 29. Krylov VN: **Phagotherapy in terms of bacteriophage genetics: hopes, perspectives, safety, limitations.** *Genetika* 2001, **37**:869-887.
 30. Sambrook J, Russell DW: *Molecular Cloning: A Laboratory Manual* New York: Cold Spring Harbor Laboratory Press, Cold Spring Har; 2001.
 31. Adams MH: *Bacteriophages* New York: Interscience Publishers; 1959.
 32. Laemmli UK: **Cleavage of structural proteins during the assembly of the head of bacteriophage T4.** *Nature* 1970, **227**:680-685.

Publish with **BioMed Central** and every scientist can read your work free of charge

"BioMed Central will be the most significant development for disseminating the results of biomedical research in our lifetime."

Sir Paul Nurse, Cancer Research UK

Your research papers will be:

- available free of charge to the entire biomedical community
- peer reviewed and published immediately upon acceptance
- cited in PubMed and archived on PubMed Central
- yours — you keep the copyright

Submit your manuscript here:
http://www.biomedcentral.com/info/publishing_adv.asp

