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Use of bacteriophages to control biofilms

Dissertation thesis for the PhD degree in Chemical and Biological Engineering

SUPERVISOR: Dr. Joana Azeredo
CO-SUPERVISOR: Professor Peter Neubauer

SANNA MARIA SILLANKORVA

Author: Sanna Maria Sillankorva
Telephone: +315253604400
email: s.sillankorva@deb.uminho.pt

Thesis title: Use of bacteriophages to control biofilms

Supervisor: Dr. Joana Azeredo
Co-supervisor: Professor Peter Neubauer

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*For my beloved son
and my parents*

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Sillankorva, Sanna, Use of bacteriophages to control biofilms

IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Braga, Portugal; Bioprocess Engineering Laboratory, Department of Process and Environmental Engineering, University of Oulu, Oulu, Finland

Abstract

After several years of abandonment, the use of bacteriophages (phages) for killing bacteria has withdrawn recent attention and reappraisal. This has led to a vast phage research, in varied fields, with impressive outcomes and currently several studies are ongoing with animals, horticulture and agriculture products, and even with humans. Despite this enthusiasm, there is a lack of research concerning phage utilization to reduce bacteria living on surfaces in a lifeform known as biofilms.

This work explores the potential of phages in controlling bacteria present in single and dual species biofilms. Gram-negative *Pseudomonas fluorescens* and Gram-positive *Staphylococcus lentus*, widespread inhabitants of dairy plant surfaces and products were the studied bacterial hosts. *P. fluorescens* is the dominant microorganism present in the microflora of raw or pasteurized milk at the time of spoilage and commonly isolated from contaminated fresh meats and refrigerated products. *S. lentus* is coagulase-negative staphylococci (CoNS) and a member of the *S. sciuri* group. CoNS are the bacteria most frequently recovered from infected bovine and caprine mammary glands.

Before performing biofilm studies, special emphasis was given to isolation of new lytic phages and to their thorough characterization. Several phages were isolated amongst which ϕ IBB-PF7A and ϕ IBB-SL58B were selected for the control experiments. Both belong to the *Podoviridae* family and can kill a wide host range. Phage ϕ IBB-PF7A resembles, morphologically, physico-chemically and genomically, to T7-like phages while ϕ IBB-SL58B cannot, so far, be assigned to any known *Podoviridae* group and is possibly a novel type of phage.

After the characterization of the phage candidates, their ability to infect biofilms was investigated. *P. fluorescens* biofilms exhibited cells with two distinct morphologies that, independently of the age of the biofilm infected, were efficiently killed by phage ϕ IBB-PF7A. Unexpectedly, the best infection by *S. lentus* phage ϕ IBB-SL58B was found to be the killing of their hosts formed under severe starvation conditions. Furthermore, biofilms

of *S. lentus* colonized better the substratum than *P. fluorescens* cells, and in effect *S. lentus* was the predominant bacterium in most dual species biofilms investigated. The dual species biofilms were challenged using two approaches. A phage cocktail, for each of the hosts of the dual species biofilms, decreased efficiently not only the cell number in the biofilm, but also the cells which were released to the planktonic phase. The use of a single phage, for the less predominant bacterium, revealed that the applied phage (ϕ IBB-PF7A) can efficiently reach the host and reduce their cell number in the biofilm, but also may cause the release of the non-susceptible species to the planktonic phase. Interestingly and contrarily to what is commonly described in literature, one of the studied phages (ϕ IBB-PF7A) proved to be excellent in killing stationary phase host cells.

The conditions under which the phages are applied are important factors to be considered. Rotary agitation and medium conditions (exchange or non-exchange) influenced vastly phage killing of biofilm cells. Additionally, rotary agitation, temperature, host cell length and growth rate influenced planktonic cell lysis by phage (ϕ IBB-PF7A). Nevertheless, the optimal conditions for phage infection are highly depended on the phage-host system investigated, as demonstrated in ϕ IBB-PF7A and ϕ IBB-SL58B infection experiments performed with their respective hosts.

Sillankorva, Sanna, Use of bacteriophages to control biofilms

IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, Universidade do Minho, Braga, Portugal; Bioprocess Engineering Laboratory, Department of Process and Environmental Engineering, University of Oulu, Oulu, Finland

Sumário

Após vários anos de abandono, assistiu-se, recentemente, ao crescente interesse científico e reconhecimento das notáveis capacidades dos bacteriófagos (fagos) no controlo de bactérias. São diversos os domínios de aplicação de fagos como agentes de controlo de bactérias, nomeadamente na produção animal, horticultura e agricultura e existem inclusive investigações a decorrer com humanos. Apesar deste recente entusiasmo, há uma grande lacuna relativamente à utilização de fagos para a redução de bactérias aderidas a superfícies ou mais propriamente biofilmes.

Este trabalho explora a utilização de fagos no controlo de bactérias presentes em biofilmes formados por uma única espécie bacteriana ou mistos (constituídos por várias espécies). As bactérias Gram-negativa *Pseudomonas fluorescens* e Gram-positiva *Staphylococcus lentus* são ubíquas em superfícies e produtos da indústria dos lacticínios. *P. fluorescens* é a bactéria dominante da microflora dos leites, não-pasteurizados e pasteurizados, estragados e frequentemente isolada de carnes e produtos refrigerados estragados. *S. lentus*, é uma bactéria coagulase-negativa (CoNS), pertence ao grupo de *S. sciuri* e pode causar doenças em animais. As CoNS são, de facto, as bactérias mais frequentemente isoladas de bovinos e caprinos com infecção das glândulas mamárias.

Antes de serem iniciados os estudos de infecção de biofilmes, procedeu-se deus-se ao isolamento de novos fagos e à sua rigorosa caracterização. Foram isolados vários fagos para *P. fluorescens* e *S. lentus* e do conjunto total de fagos purificados, os fagos ϕ IBB-PF7A e ϕ IBB-SL58B, foram seleccionados para os estudos de controlo de biofilmes. Ambos pertencem à família *Podoviridae* e têm um espectro lítico alargado. O fago ϕ IBB-PF7A assemelha-se morfológicamente e genomicamente a fagos do tipo T7, enquanto que o fago ϕ IBB-SL58B poderá ser um novo tipo de fago uma vez não há semelhanças morfológicas, genómicas nem físico-químicas com nenhum fago descrito na literatura.

O fago ϕ IBB-PF7A foi capaz de lisar com grande eficiência os biofilmes de *P. fluorescens*, independentemente da idade do biofilme. Inesperadamente, este estudo revelou que a melhor infecção dos biofilmes de *S. lentus* por parte do fago ϕ IBB-SL58B foi conseguida em biofilmes formados sem renovação do meio de crescimento. No que respeita à infecção de biofilmes mistos, formados por *P. fluorescens* e *S. lentus*, utilizou-se duas estratégias distintas: o uso de um cocktail formado pelos dois fagos e a aplicação de apenas um fago específico para a bactéria minoritariamente presente nos biofilmes mistos, a *P. fluorescens*. A primeira estratégia revelou uma redução no número de células viáveis presentes nos biofilmes e de células libertadas dos biofilmes para a fase planctónica. A utilização de apenas um fago foi eficaz no controlo das células de *P. fluorescens* presentes no biofilme, assistindo-se a uma destruição parcial do biofilme e consequentemente à libertação para a fase planctónica da bactéria não susceptível ao fago. Contrariamente ao que é frequentemente descrito na literatura, um dos fagos estudados (ϕ IBB-PF7A) revelou uma elevada eficácia na lise de células na fase estacionária de crescimento.

A avaliação de diferentes condições de infecção é fundamental de forma a se conseguir otimizar a eficiência fágica no controlo de biofilmes e células planctónicas. Este trabalho evidencia que a aplicação de fagos no controlo dos hospedeiros é influenciada por factores tais como: a velocidade de agitação, temperatura e vários parâmetros relacionados com o hospedeiro tais como o seu tamanho, taxa específica de crescimento e com as condições usadas na formação de biofilmes nomeadamente a renovação ou não de meio de crescimento. No entanto, este trabalho revela também que as condições óptimas de infecção fágica dependem bastante do fago e hospedeiro investigados como verificado em ensaios de infecção realizados com os fagos ϕ IBB-PF7A e ϕ IBB-SL58B e os seus respectivos hospedeiros.

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Abbreviations

Abbreviation	Extension
bp	Base pair
CFU	Colony forming units
CLSM	Confocal laser scanning microscope
DNR	Dynamic biofilm formation conditions with non-renewal of media
DR	Dynamic biofilm formation with media renewal
EM	Electron microscopy
EPS	Extracellular polymeric substances
FESEM	Field emission scanning electron microscope
gp	Gene product
IBB	Institute for Biotechnology and Bioengineering
ICTV	International committee on the taxonomy of viruses
FDA	Food and Drug Administration
MOI	Multiplicity of infection
nt	Nucleotide
OD	Optical density
PBS	Phosphate buffer saline
PEG	Polyethylene glycol
PFU	Plaque forming units
SD	Standard deviation
SR	Static biofilm formation with media renewal
SS	Stainless steel
TEM	Transmission electron microscope
TSA	Tryptic soy agar
TSB	Tryptic soy broth
USDA	United States Department of Agriculture

INTRODUCTION

Pathogenic and spoilage bacteria are consistently found living in sessile communities attached to a wide range of biotic and abiotic surfaces. These communities, better known as biofilms, are accountable for spoilage of products in food, dairy and food processing industry, and can cause transmission of diseases. Biofilms tolerate 100 to 1000-fold higher levels of antimicrobial agents than planktonic cultures and, although several strategies are adopted to prevent biofilm formation and their removal, biofilms do persist in a wide range of industrial surfaces. This reveals an inefficacy of the cleaning and disinfection mechanisms and demands a quest for possible alternatives.

Bacterial viruses or bacteriophages (phages) are hypothesized to be the predominant lifeform in the biosphere, clearly outnumbering their host bacterium. Phages are currently suggested as possible alternatives to antibiotics for the treatment of bacterial diseases in humans and animals and widely explored to minimize the pathogen loads in food products of animal and plant origin. Thus, why couldn't phages also be used as an alternative to industrial disinfectants and sanitizers, avoiding the undesirable effects of the vast doses of antimicrobial agents used to rid biofilms from surfaces? After all, phages do have a variety of advantages over chemical agents. Their isolation is fast and simple. Their production is inexpensive. They are specific against a host or host range and thus do not affect the normal microflora of the environment where they can be applied. They are considered environmentally friendly. They self-replicate at the infection site as long as the host bacterium is present and, so far, no serious side effects have ever been reported.

The present study was carried out to further understand the potential of phages to reduce bacterial biofilms from surfaces and to scrutinize if they could really provide an alternative in industrial environments. For this, the specific aims of this thesis were:

1. to isolate, select and characterize phages.
2. to investigate the chosen phages for their ability to function as biological control agents and to further widen the knowledge on how phages control single and dual species biofilms.
3. to study phage killing of planktonic cells varying cell growth and phage infection parameters.

This thesis is composed of 6 different chapters which include original articles and unpublished work.

Chapter 1 provides a general background of phages and biofilms and includes an overview of phage applications carried out in food and food processing environments.

In Chapter 2, the isolation of new phages and their thorough characterization is described. Characterization includes morphological, physico-chemical, growth cycle and adsorption evaluation of the phages. Additionally, the complete genomic characterization of *Pseudomonas fluorescens* phage ϕ IBB-PF7A and the preliminary genome characterization of *Staphylococcus lentus* phage ϕ IBB-SL58B are presented.

Chapter 3 describes studies on the ability of phages to control biofilms. The effect of phage ϕ IBB-PF7A and ϕ IBB-SL58B on single species biofilms of *P. fluorescens* and of *S. lentus* are studied varying biofilm formation and phage infection parameters. Characterization of dual species biofilms of *P. fluorescens* and *S. lentus* formed under three distinct conditions and their challenge using two phage treatment approaches is also described.

Chapter 4 presents the studies of phages on *P. fluorescens* planktonic cultures. The effect of some parameters on cell morphology and on phage infection is described in this Chapter.

Finally, Chapter 5 presents the main conclusions extracted from the work presented in this thesis and a few recommendations for future work.

1. LITERATURE REVIEW

2. ISOLATION AND CHARACTERIZATION OF PHAGES

3. PHAGE BIOFILM INTERACTION

4. PHAGE AND PLANKTONIC CELLS

5. MAIN CONCLUSIONS AND SUGGESTIONS FOR FORTHCOMING WORK

1.1 Bacteriophages

Bacterial viruses were discovered approximately 100 years ago [1-3] and their discovery is equally attributed to Frederick Twort, an English bacteriologist, and Félix d'Hérelle, a French-Canadian microbiologist [4, 5]. However, Twort didn't continue his research and it was d'Hérelle who seriously pursued working with these viruses and named them, in 1916, as "bacteriophages" from the words "bacteria" and "phagein", which in Greek means to eat or devour. d'Hérelle performed numerous trials in humans and animals (rabbits, chicken and buffaloes) [6] trying to shed a light on their nature and ability to function as therapeutic agents [1, 5]. He isolated bacteriophages (phages) for a number of bacterial hosts causing diseases such as: cholera, diphtheria, bubonic plague and anthrax. There was also a time when phage products were manufactured in large scale at d'Hérelle's laboratory in Paris (today known as L'Oréal), and by several existing drug giants (Eli Lilly & Co, Parke-Davies, Squibb & Sons and Swan-Myers division of Abbott Laboratories, etc.) [7-9]. Twenty years after the official finding of phages, the first antibiotic, penicillin, was discovered. This fact, allied with some early clinical failures [10] and theoretical concerns led to the abandonment of phage therapy in U.S. and most of the Western Europe. Nevertheless, research and therapeutic use of phages persisted in the former Soviet Union and Eastern European countries [11-14], where phages continued to be regarded as a good treatment method against a wide range of bacterial infectious diseases [8, 15].

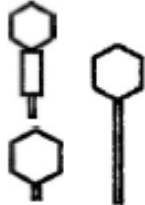
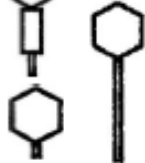
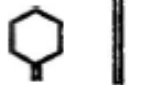










1.1.1 Isolation, classification and prevalence

It has been hypothesized that wherever bacteria thrive it is possible to find at least one phage for each different host. Soil, water, sewage, humans' and animals' body (skin, oral cavity, saliva, faeces, gut) [16-19] and even the food we eat [20] are just some examples from where phages have been isolated. Phages have evolved to survive harsh environments like extreme temperatures [up to 95°C (hotsprings)] and pH values as low as 1.3 [21].

There is a diversity of phages spread worldwide and a vast array of shapes, sizes, capsid symmetries and structures. Their genomes can be either DNA or RNA and single stranded (ss) or double stranded (ds). Phage classification has been assigned to the International Committee for Taxonomy of Viruses (ICTV) that organizes phages into

different families of the *Caudovirales* order (Table 1.1). The ICTV classification is based mainly on morphological analysis, nucleic acid type and host organism. However, other properties, regularly updated by ICTV, such as: physico-chemical, biological, protein amount and size, lipid content and characteristics, among others standards, are equally used for phage classification.

Table 1.1 – ICTV classification of *Caudovirales* phages.

Family	Nucleic acid	Characteristics	Morphology
<i>Myoviridae</i>	Linear dsDNA	Non-enveloped, contractile tail	
<i>Siphoviridae</i>	Linear dsDNA	Non-enveloped, long non- contractile tail	
<i>Podoviridae</i>	Linear dsDNA	Non-enveloped, short non-contractile tail	
<i>Corticoviridae</i>	Circular dsDNA	Non-enveloped, isometric	
<i>Tectiviridae</i>	Linear dsDNA	Non-enveloped, isometric	
<i>Lipothrixviridae</i>	Linear dsDNA	Enveloped, rod-shaped	
<i>Plasmaviridae</i>	Circular dsDNA	Enveloped, pleomorphic	
<i>Rudiviridae</i>	Linear dsDNA	Non-enveloped, rod-shaped	
<i>Fuselloviridae</i>	Circular dsDNA	Non-enveloped, lemon shaped	
<i>Inoviridae</i>	Circular ssDNA	Non-enveloped, filamentous	
<i>Microviridae</i>	Circular ssDNA	Non-enveloped, isometric	
<i>Leviviridae</i>	Linear ssDNA	Non-enveloped, isometric	
<i>Cystoviridae</i>	Segmented dsRNA	Enveloped, spherical	

It is estimated that 96% of all phages in the world belong to one of the three groups of tailed phages. These three phage groups essentially differ in the tail type they possess – contractile or non-contractile and long or short. Among the less common phage families, some formed by only one element, are phages with polyhedral, filamentous, and pleomorphic morphology.

The ICTV taxonomical system requires always electron microscopy (EM) visualization however this doesn't allow the classification of the numerous prophage genomes that are found within the sequenced microbial genomes. Recently, a new

strategy for phage classification has become popular and relies on sequencing of the phage genomes. A phage proteomic tree has been assembled with data of several sequenced phage genomes and this tree organizes phages relative to both their near neighbors and to all other phages in the database [22]. All predicted protein sequences present in the genomes are extremely useful for phage grouping, independently, whether the common protein pool occurred from a common ancestor or via lateral transfer. The proteins, which are conserved in all members of a specific group, can be regarded as genetic markers for their respective groups and therefore can be used to classify numerous unknown representatives in the environment and allow their distribution within the distinct groups of the phage proteomic tree. However, some discrepancies between the two classification strategies, the genome-based and the morphological taxonomic classification by ICTV, have been discovered. For instance, *S. typhimurium* phage ϕ P22, a *Podoviridae* according to ICTV standards, revealed resemblance, at genomic level, to Siphophage λ -like phages. Also, the enteric phage ϕ PRD1 described as *Tectiviridae* (ICTV) groups genomically with the PZA-like Podophages [22].

The estimated global phage population size is extraordinarily high. For instance, it is presumed that aquatic habitats have total phage numbers above 10^{31} [16, 23]; terrestrial ecosystems have revealed 10^7 viruses per gram of soil [16] and sewage present total phage numbers in the range of 10^8 - 10^{10} per milliliter [24]. It has also been shown that phages clearly outnumber bacteria in essentially all studied environments [16, 18, 20] and are hypothesized to be the predominant lifeform in biosphere.

1.1.2 Life cycles

Phages, like all other viruses are obligate intracellular parasites and thus, in order to replicate they require a specific host. Outside of a host, phage particles are just inert packages that protect the genome from physical, chemical and enzymatic damage until the vital nucleic acid is delivered into a susceptible host. Phages carry their genetic information either in the form of DNA or RNA. Phage contact with a host bacterium occurs via their tails and tips that recognize the necessary receptors (carbohydrate, protein and lipopolysaccharide molecules and flagella) that are present on the hosts' surface. Most phages are highly specific for their receptors and there is poor or no interaction with receptors with slightly different structures. This high specificity is the basis of phage

typing methods that are widely used for the identification of bacterial species or subspecies [25].

Lytic phage infection

The lytic phage pathway starts when the virion interacts with the hosts' cell surface receptor molecules. After phage adsorption to these molecules, the cell wall is made penetrable and the nucleic acid is transported into the cell, whereas the capsid remains outside the cell. Inside the host occur several steps which include gene expression, genome replication and morphogenesis – i.e. the formation of the capsids (and tails) and the packaging of the genomes into the capsids (Figure 1.1). Phages are reproduced very quickly, forming new virion particles and this reproduction phase ends with the lysis of bacteria. With the host lysis, hundreds of new phages are released from each infected bacteria [26]. The number of new phages produced, or progeny, depends on the species and conditions, nevertheless each “parent” phage is able to produce in average 50 - 200 “daughter” phages per lytic cycle [27]. Lytic phage infection results in clear plaques on the respective host bacterial lawns.

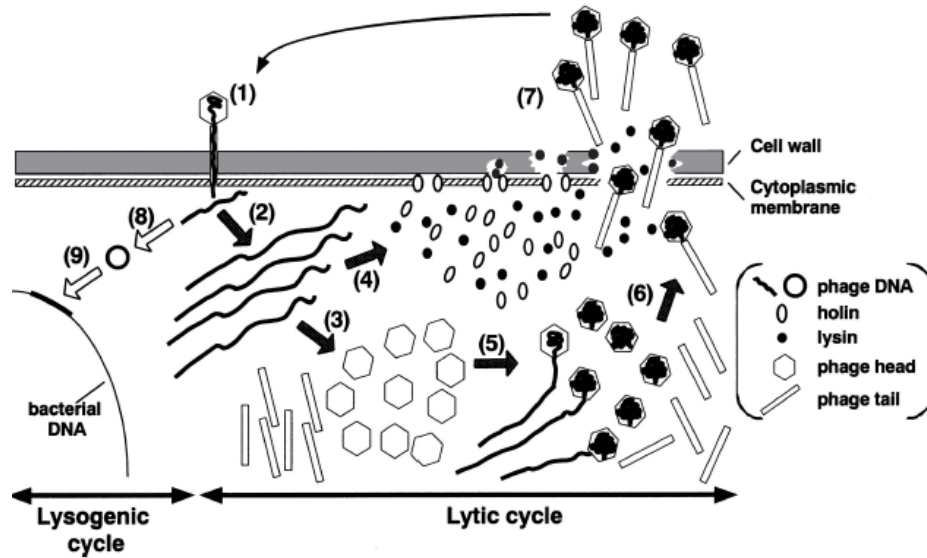


Figure 1.1 - Lytic and lysogenic life pathways. (1) adsorption and DNA injection; (2) DNA replication; (3) head and tail production; (4) synthesis of holin and lysin; (5) DNA packaging; (6) disruption of the cell wall and release of the progeny; (7) circularization of phage DNA; (8) circularization of phage DNA; (9) integration of the phage DNA into the host genome (from Matsuzaki *et al.*, 2005).

Lysogenic phage infection

Phages with lysogenic growth integrate their prophage into the chromosome or other replicon of the host bacteria. This results in a so called lysogenic infection where the phage genome is passed, through cell division, to all daughter cells (Figure 1.1). Phages with this type of growth cycle form turbid plaques on the susceptible host bacterial lawns. When a strain is lysogenised with a phage, it becomes resistant to infection by other related phages that share the same immunity group profile or same repressor specificity [28]. Some phages represent their prophages as plasmids that are replicated and that are allocated to the bacterial daughter cells in strict accord with bacterial replication and division. From time to time, the level of repressor concentrations decreases and in these cases the transcription of genes whose products are required for the lytic pathway is activated. This causes the formation of the phage progeny and cell lysis (spontaneous induction). These virulent mutants of temperate phages are not always distinguished from virulent phages and do not respond to the presence of a specific cell receptor [29].

1.1.3 Polyvalent and gene-engineered phages

Polyvalent or broad-host range phages can productively infect a variety of bacterial host species or strains. In complex natural communities (ex. biofilms), the opportunities for an effective viral reproduction are increased when phages can profit from a more varied potential host range [30]. There is a diversity of polyvalent phages for distinct host bacterium described in literature (Table 1.2). This illustrates that polyvalent types of phages can be readily isolated from complex natural microbial communities. These polyvalent phages can be extremely useful in animal therapy and prophylaxis or even for reduction of pathogen or spoilage bacterial loads in food products as one single phage will be able to lyse more strains than a non-polyvalent phage. Nevertheless, in practice these wide host range phages will most likely disappear after several applications, due to the emergence of phage-resistant bacterial mutants.

Table 1.2 – Examples of polyvalent phages described in literature.

Bacteria	Polyvalent phage name (reference)
<i>A. aerogenes</i> , <i>E. coli</i> , <i>K. Pneumoniae</i>	φmp [31]
<i>Campylobacter</i>	CP8, CP34 [32]
<i>Enterococcus faecalis</i>	φEF24C [33]
several enteric species + <i>E. coli</i>	P1 [34]
<i>E. coli</i> , <i>mycobacterium</i>	P1 [35]
<i>E. coli</i>	Mu [36]
<i>E. coli</i> , <i>Klebsiella</i> , <i>Salmonella</i>	[37]
<i>E. coli</i> , <i>P. aeruginosa</i>	BHR1, BHR2 [30]
<i>E. coli</i> , <i>S. natans</i>	BHR3, BHR4, BHR5 [30]
<i>Salmonella</i>	O-1 [38]; st104a and st104b [39]
<i>S. natans</i> , <i>P. aeruginosa</i>	SN-1, SN-2, SN-X, SN-T [30]
<i>Staphylococcus</i> spp.	P14, S3K, Muscae [40]; 06, 40, 58 [41]; Gratia [42]; A/3, A/5, f200, X, PK [43]; Sb-1 [44,45]; K [46,47]; A, EW, J10, J11, K1, K2 [48]; P1 [49]; φ812, SK311, φ131 [50]
<i>V. vulnificus</i>	A-9, J-7, VBNO, 304C, MO6-24 [51], 71A-6 [52]

There is a surprisingly large number of polyvalent phages reported for *Staphylococcus* spp. (Table 1.2) and it is particularly remarkable that they all belong to the same family of phages – the *Myoviridae* [53]. Novick suggests that all these polyvalent staphylococcal phages adsorb to the same receptor which is located in the peptidoglycan–teichoic acid complex in the cell wall [54]. Thus, the differences in host susceptibility are only related to post-adsorption phenomena.

Gene-engineering phages is attracting worldwide attention and will most likely become an integral part of phage therapy. This field of phage research is still recent, however, astonishing results have been reported. For instance, T4 phage has been modified by replacing the tail fibers to change the adsorption specificity of the phage [55, 56]. This modification increased the host range, increasing the potential of phage application. Filamentous phage M13 has been improved and modified to suppress *Helicobacter pylori*'s growth and to deliver antimicrobial agents to *E. coli* bacteria [57,

58]. Another example of a gene-engineered phage was described by Hagens *et al.* (2004) that succeeded in producing a stable, efficient, and safe therapeutic phage for *P. aeruginosa* through modification of phage Pf3R in order to minimize the hazardous effect caused by endotoxin release [59]. Modified filamentous phages (f1.K) can also serve as an alternative to traditional carrier proteins for synthetic peptides, carbohydrates and haptens [60]. Lu and Collins improved the characteristics of T7 phage to better attack biofilms by modifying the phage to express a biofilm-degrading enzyme, while the phage replicates inside the host. The refactored T7 phage can thus simultaneously attack the bacterial biofilm cells and degrade, in large extent, the polymeric matrix of biofilms [61].

Besides filamentous phages, the T-odd phages of T7 and T3 type are the most likely candidates for gene engineering. These phages are represented in various bacterial species and families (ex. *Escherichia*, *Pseudomonas*, *Klebsiella*, *Serratia*, *Citrobacter*) and once the phages have suffered modification they will probably allow the expression of killing genes in various bacterial species. Also, T7 and T3 have small genomes and high rates of phage development. Their small genome size are an advantage as they will not allow the transfer of extended fragments of chromosome DNA carrying several pathogenity islands [29].

1.1.4 Bacterial resistance to phages

Bacteria can become resistant to phages when: a disturbance occurs during the phage development, through specific mechanisms to protect themselves against the invasion of foreign DNA, genetic exchange with other bacteria, acquisition of some plasmids, and lysogenic conversion [29, 62]. Disturbance during phage development can occur through loss or change by mutation of the hosts' receptor molecules to which phages adsorb. In these cases, cells become refractory to a phage that used to be able to infect them [29,62]. Disturbance can also occur after phage genome injection into a host by its degradation with bacterial DNases at different stages of intracellular biosynthesis (replication, transcription, translation, phage particle maturation), and, finally, because of the blocked lysis and progeny release [29]. This phenomenon is named restriction-modification immunity [63].

Bacterial protection against the invasion of foreign DNA is based on the modification of the host's DNA by methylation. This modification occurs at specific points on the DNA sequence which concomitantly will give protection to cleavage by host specific

restriction endonucleases. Host-mediated restriction results in the cleavage of all foreign DNA which does not carry the corresponding methylation pattern. Some unmodified phage genomes avoid host-mediated restriction and, on being replicated, become modified. This enables them to evade restriction by a particular host restriction/modification system in subsequent infective cycles [21]. In any circumstances, the development of cross-resistance has never been reported. Thus, a bacterium that becomes resistant to a specific phage will still remain sensitive to other phages (ex. *E. coli* resistant to λ is still sensitive to T7).

There are also situations in which the evolution of phage-resistant bacteria could be positive. The host receptors to which phage adsorb can be capsules or other virulence determinants and the development of phage resistance would immediately reduce virulence [58, 64-66]. In these cases, resistance to phages would create mutant bacteria that would no longer be capable of causing disease. Furthermore, as long as phages are present as a selective agent, the resistant, avirulent mutants would probably replace the virulent forms. Thus, phages could even be chosen specifically for this property.

1.2 Bacterial biofilms

Direct observations of microbial biofilms have been reported since the first microscopes were built. For instance, the Dutch researcher Antoine van Leuwenhoek (1632-1723) observed this type of lifeform when examining the “scuff” from his teeth using a self constructed microscope capable of enlargements of up to five hundred times. Biofilms are found in extreme environments such as hydrothermal vents, nuclear power plants [67], oil-recovery industries and even desert boulders [68].

Since the first biofilm observations, the definition of biofilm has changed considerably. The most recent definition states that a biofilm is a multicellular community composed of prokaryotic and/or eukaryotic cells embedded in a matrix composed, at least partially, of material synthesized by the sessile cells in the community [68].

In nature, many species of bacteria, fungi, protozoa and algae form biofilms. However, bacteria have received the most attention and it is estimated that 99% of the bacteria present in natural and pathogenic ecosystems can grow in these metabolically integrated communities.

1.2.1 Biofilm formation stages

The formation of a biofilm requires a series of discrete and well regulated steps (Figure 1.2). Biofilm formation begins with adsorption of macromolecules (proteins, polysaccharides, and humic acids) and smaller molecules (fatty acids and lipids) at surfaces. Adsorbed molecules form conditioning films that alter physiochemical characteristics of the interface, including surface hydrophobicity and electrical charge. After the surface conditioning step, microorganisms start to adhere. Adhesion can roughly be divided into two phases: the reversible and the irreversible. The reversible phase essentially includes long-distance interactions such as electrostatic, hydrophobic interactions and Van der Waals forces while in the irreversible phase are various short range forces (dipole-dipole, hydrophobic, ion-dipole, ion-ion, covalent bonds and hydrogen interaction). The predominant surface colonizers are planktonic bacteria. Nevertheless, aggregates of cells detached from biofilms can also start biofilm re-growth when trapped in surface irregularities.

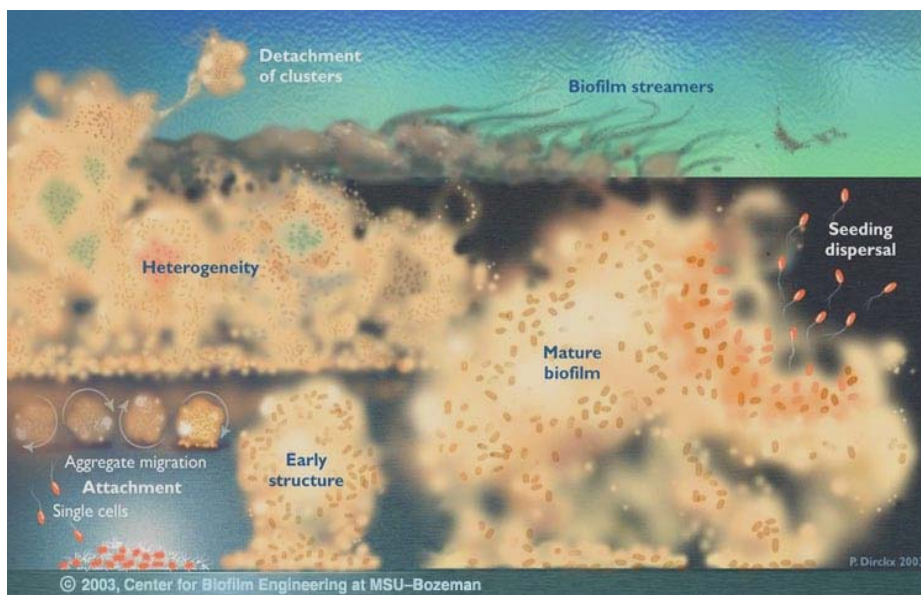


Figure 1.2 - Biofilm formation steps, from attachment of single cells, maturation of the biofilm and release through cluster detachment and seeding dispersal (reprinted with permission of the Center for Biofilm Engineering, Montana State University, Bozeman, U.S.A).

Cells adhered to surfaces start modifying their planktonic gene expression to the biofilm phenotype to suit the precise micro-niche in which they find themselves [69].

Adherent cells also change the physical connections they have with the surface and with neighboring cells by the secretion of polysaccharides and other matrix components (Figure 1.2). Furthermore, cells can change their phenotype and even the position in the community in order to reach a scenario of better survival possibility. In monospecies and mixed-species biofilms, the position of individual bacterial cells does not occur randomly. It appears that some undetermined species-specific rules and certain classes of *pili* can dictate cell positioning. The volume and extent of biofilms are fully dependent on the nutritional and environmental factors present (pH, temperature, nature of biotic and abiotic surface, etc.) and the attachment and biofilm formation by microorganisms is influenced also by signals from adjacent cooperative and competitive bacterial species that influence cell positioning and the activity in the community [68, 70].

When cells detach from biofilms, mobile post-adhesion behaviors can be observed, during which cells will preferably associate with cells of the same and metabolically cooperative species where they will start forming aggregates. Detachment depends of factors such as biofilm thickness, fluid shear stress, nutrient availability, and fluid velocity [70].

There are no limits to the variety of cellular arrangements in real biofilms. The reported biofilm architectures are very diverse, from the tower mushroom and water channel structures, to tower, bridges and rosettes [71], stump and mushroom cap architectures [72]. Cells can move within the biofilms and causing a distinct biofilm configuration.

1.2.2 Advantages of living in a biofilm community

Microorganisms adopt the biofilm mode of growth as a survival strategy to resist against environmental stresses and inactivation caused by conventional methods such as antibiotics and disinfectants, which are otherwise effective against free-floating cells [73-83]. The success of an antimicrobial agent is dependent upon its ability to inactivate and remove biofilm organisms. Several factors influence biofilm tolerance to antimicrobial compounds. For instance, common antimicrobial agents are produced for fast-growing the bacteria and thus bacteria on the center of a microcolony are spared as they are slow-growing for there is less oxygen and nutrients available. Also, intercellular signals can alter the physiology of the biofilms and this causes members to produce molecular pumps that expel antibiotics from the cells and allow the community to grow even in the

presence of a drug. Moreover, positively charged antimicrobials can bind to the negatively charged biofilm matrix and this prevents the agents from reaching the cells within a colony. Specialized persister cells do not grow in the presence of an antibiotic however, these cells are not killed. Furthermore, the population diversity can improve the survival chances of some cells [84]. Thus, the inherent resistance of bacteria in biofilms can lead to cycles of biofilm re-growth after the system disinfection procedures.

Besides protection against antimicrobial agents, there are other advantages of living in a biofilm community such as: increased availability of nutrients for growth; increased binding of water molecules, reducing the possibility of dehydration; proximity to progeny and other bacteria, facilitating plasmid transfer [70].

1.2.3 Impact of biofilms in food and dairy industry

In most food and dairy industries, pathogenic and spoilage bacteria tend to attach to equipment surfaces and form biofilms. These biofilms are important reservoirs of microbial contamination that may lead to equipment damage, energy losses, spoilage of finished products and transmission of food pathogen that may cause diseases. Bacterial biofilm formation in food industries has been the focus of some reviews [79, 85, 86] but still has not received enough attention within the food and dairy processing areas. Biofilms tend to form on the surfaces of equipment used for example in food handling, storage, or processing, especially in sites that are not easy to clean or sanitize (ex. dead ends, joints, corners, valves, and gaskets in tubing systems). Corroded areas of equipment surfaces are also ideal places for the development of biofilms. Besides stainless steel and Teflon, which are common equipment materials in industrial environments, biofilms are also found on a diversity of packaging and other equipment surfaces such as plastic, rubber, glass, wood etc. and they can exist also in food products [87].

During industrial process operation it is often difficult to detect where the biofilms reside, which makes biofilm treatment a challenging task. It is therefore important to understand the interactions between biofilms and the surfaces they are found on, in order to design adequate cleaning and disinfection methods to provide more effective measures for biofilm removal and prevention [86]. Nowadays, limiting the growth of microbes is accomplished through: a good production hygiene, a rational running of the process line, and a well designed use of biocides and disinfectants [88]. Effective cleaning and disinfection should remove all undesirable materials from the surfaces (microorganisms, soil, foreign bodies and cleaning chemicals). The cleaning steps usually involve wetting

of the soil and surfaces with the cleaning agent(s); reaction of the chemical agent to facilitate removal from the surface; prevention of re-deposition and disinfection of residual microorganisms [89, 90]. However, the presence of biofilms is highly prevalent and difficult to completely eliminate. Biofilms have been found even on surfaces where acceptable cleaning procedures have been employed [89, 91-94] which compromises the sanitation standards of food processing environments.

1.3 Phage applications

In recent years, there has been an increase of bacterial resistance to one or a number of antimicrobial agents. This has once again drawn attention to phage therapy and to a re-appraisal of this biological therapeutic alternative for killing pathogenic bacteria. The procedure for using phages as therapeutic agents is rather simple and phages have many advantages over antimicrobial agents such as: specificity against a host or host range not affecting the normal microflora; self-replication capability at the infection site, as long as the host bacteria is present; no serious side effects have ever been reported, the production is simple and inexpensive and phages are environmentally friendly.

Human therapy

Nearly since phage discovery, these biological agents have been continuously applied to humans in a small number of countries (Georgia, Poland, Russia). Phages are used to treat several diseases such as abscesses, suppurating wounds, vaginitis, acute and chronic infections of the upper respiratory tract, etc. Human therapy with phages has been performed using phages alone, combining them with other agents such as antibiotics or as a last measure after the failure of antibiotics therapy [95-99].

Recently, human trials are ongoing in UK and USA. In the South West Regional Wound Care Center, Lubbock, Texas in USA, phase I trials are being carried out against *P. aeruginosa*, *S. aureus* and *E. coli* and in the Great Ormond Street hospital in London, UK, phase II phage trials are being done against otitis infections caused by *P. aeruginosa*.

Food industry

Food industry faces a variety of challenges to keep products safe and free of pathogenic microorganisms for the sake of the consumers and the product itself. However, annually, there are numerous reports of illness, hospitalization and even death

of people due to a variety of food-borne pathogens present on the food we eat. There are more than 200 different known food-borne pathogens including viruses, bacteria and parasites that can cause food-borne illness, along with toxins, chemical contaminants and metals. Since some years, the use and research on the field of natural antimicrobial agents has increased due to alterations in consumer positions towards the use of antibiotics and synthetic preservative agents in food, surface detergents and disinfectants which are amongst the leading causes of the increased emergence of antimicrobial resistant bacteria.

Also, due to consumer demands and trends in the food industry, it is nowadays possible to buy ready-to-eat food (e.g. bagged salads, fresh-cut fruits, prepared foods, frozen dishes, etc.), that have greatly challenged the food production technologies and demanded new strategies for the prevention of food-borne pathogens in the products. When food safety measures are not strictly used, mixing and handling of enormous volumes of products can increase the spread of microbial contaminants, when present, leading to far more episodes of illness. Many fresh-cut produces, acquired on the markets, are processed in industrial facilities where cross-contamination with pathogenic bacteria from different sources can occur.

So, recently phages have been studied and applied to food products of animal (Figure 1.3) and plant (Figure 1.4) origin.

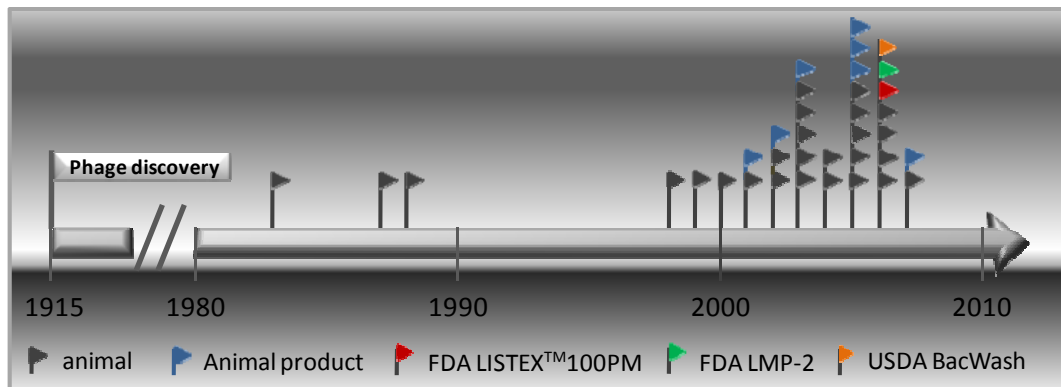


Figure 1.3 - Timeline of phage application in animal production or to foods of animal origin.

Animals such as poultry, fish and livestock have been infected with phages in order to decrease the population of pathogenic bacteria and attempt to minimize animal disease [32, 100-109]. The vast majority of the studies reported in this field occur in poultry industry where the animals and products (meats, eggs) get frequently contaminated with *E. coli*, *Salmonella* and *Campylobacter*. Many of these studies report successful

reductions of these pathogens loads with different phages [32, 65, 66, 102-106, 109 - 110]. For instance, phage treatment of chickens and calves has shown to protect the animals against septicemia and meningitis caused by *E. coli* and to reduce morbidity of the treated animals compared to control experiments [103-106, 111-115]. Phages also showed to delay the appearance of this bacterium and by this mean lengthened the animal life period [116]. Chickens treated with *Campylobacter jejuni* phages showed a decrease in the amount of this pathogen in the skin and caecal content and resulted in a delay of *C. jejuni* colonization. The same observations have also been described with *Salmonella* phages [117] and a decrease of this pathogens' recovery from the carcass rinsing waters due to phage application [102]. In animal husbandry, phages can be applied at different stages of processing and in all areas where animals contact. Phages have also been successfully applied to ruminants (steer, sheep) [118,119], have decreased the mastitis episodes by 16.7% in Holstein cows [120], and even have decreased hemorrhagic ascites disease and septicemia in ayu and yellowtail fish [108, 121]. Phages have also proved to significantly reduce the pathogen loads in cheese [122, 123], frankfurters [124] and even in infant formula [125].

Plant pathogens are rarely dangerous for humans; however they are one of the main causes of product loss. Plant pathogens such as *Ralstonia solanacearum* and *Pseudomonas syringae* cause bacterial wilt, foliar spots and blights and affect a variety of products (potato, tobacco, tomato, banana, peanut and soybean). Phage research is occurring in horticulture and agriculture (Figure 1.3) and it aims at protecting plants, vegetables and fruits from decay and spread of bacterial diseases.

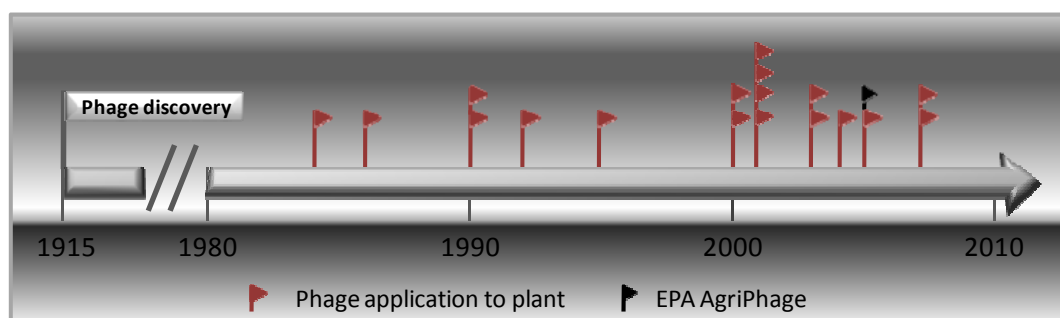


Figure 1.4 - Timeline of phage applications in agriculture, horticulture or to foods of plant origin.

The main products to which phages have been applied to are: geranium [126,127], tobacco [128], tomato [129, 130], potato [126, 130, 131], mushroom [132], sprouts [133]), peach [134] and apple [135]. However, phage application can be compromised

due to environmental factors such as sunlight irradiation, especially in the UV zone, temperature, desiccation, and exposure to copper bactericides due to their ability to destroy the phages applied in the phyllosphere. Studies show that for a successful application there are a number of steps that can be carried out. For instance, to mitigate the phage population decrease due to UV irradiation exposure, phage applications to tomato fields should always be performed in the the early morning, midmorning, early afternoon, and late evening. Phage formulas can also be created by mixing phages with other components (e.g. skim milk) protecting phages from ambient temperature alterations. Non-protected phage formulas were reduced by desiccation after 60 days and by fluorescent light after 2 weeks. However, both of these factors had little effect on phages mixed with skim milk [126, 129, 130, 136], which shows that phage formulas have a high potential as plant disease control agents.

The vast research carried out with phages and some food products of plant and animal origin has led to the fabrication of phage or phage-based products. Today, in animal production, animal and plant products, the application of three four products is allowed. Two of these phage products, LISTEX™ 100PM and LMP-2, target *Listeria monocytogenes* present in cheese, meat and fish products and both have been approved by the US Food and Drug Administration (FDA). Additionally, LISTEX™ 100PM has also received the Generally Recognised as Safe (GRAS) status by the FDA and by the United States Department of Agriculture (USDA), to be incorporated in food production processes. Another phage product named BacWash™, has been issued a No Objective Letter For Use, by the USDA, and it targets *Salmonella* and *E. coli* O157:H7. BacWash™ phage was especially created to be used on animals prior to slaughter and it can be applied as a wash, mist, or sprayed directly to the live animal. The Environmental Protection Agency (EPA) approved, in 2005, the product AgriPhage™ for control purposes of *Xanthomonas campestris* pv. *vesicatoria* and *Pseudomonas syringae* pv. in tomato, and pepper plants among others. This product can decrease bacterial spot and speck and it can be used on farms at the pre-harvest level, diluted prior to dispersal and can be applied to crops by different means, such as drip irrigation or using ground or aerial spray equipment.

Biofilm-phage interaction studies

It is a known fact that biofilms are the predominant bacterial lifestyle in surfaces. However, most research with phage is being performed with their planktonic counterparts and not with biofilm communities. Since phage discovery, in the beginning of the 20th century, the first phage-biofilm study was reported only in 1995 (Figure 1.5).

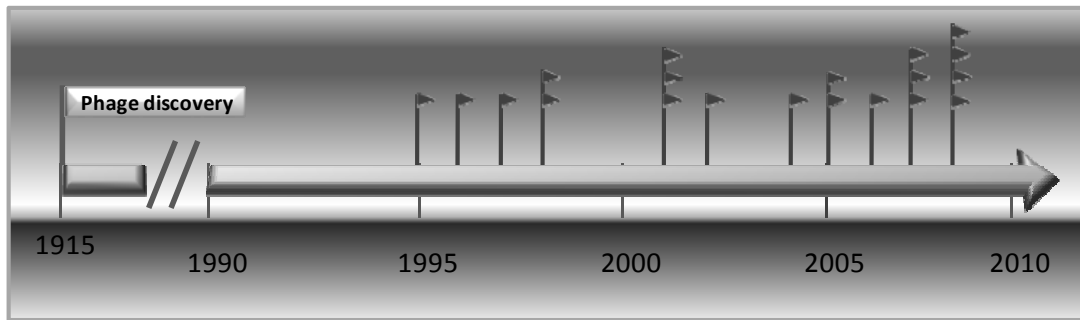


Figure 1.5 – Timeline of studies on interaction of phages with bacterial biofilms.

Different phages have been used to infect a variety of bacterial biofilms (Table 1.3) and in general, all these phage-biofilm interaction studies reveal that phages are capable of decreasing the bacterial populations.

The treatment of biofilms using phages is a complex process and only strictly lytic phages should be used. Like in phage infection of planktonic cells, there are several essential steps that need to occur. The first and crucial step in phage infection is the adsorption of phages to the receptors of the target bacteria. The EPS matrix, in which bacteria are embedded in, can constitute a problem for phages, as it needs to be penetrated so that phages can reach and adsorb to the specific receptors located on the target hosts' surface. However, it has been reported that phages are well capable of penetrating through the EPS matrix by diffusion or due to the presence of phage associated enzymes. These enzymes have the role of destroying the matrix so that the phages can get in contact with lipopolysaccharides, outer membrane proteins or other receptors necessary for the start of the host infection [137, 138]. The activity of polysaccharide depolymerase enzymes has been reported in biofilms of *E. agglomerans* infected with phage SF153b and also hypothesised, based on the visible degradation observed, in *P. fluorescens* biofilms infected with phage ϕ S1 [131].

Table 1.3 – Phages and bacterial hosts used in biofilm infection experiments.

Year	Bacteria / phage	Phage	Reference
1995	<i>E. coli</i>	T4	[139]
1996	<i>E. coli</i> , <i>P. aeruginosa</i>	T4, E79	[140]
1998	<i>E. agglomerans</i> 53b	SF153b	[137]
1998	<i>E. agglomerans</i> Ent and 53b	SF153b, ϕ 1.15	[138]
2001	<i>E. coli</i> K-12	T4	[141]
2001	<i>P. aeruginosa</i>	F116	[142]
2001	<i>S. epidermidis</i>	?phage	[143]
2002	<i>E. cloace</i> and <i>E. agglomerans</i>	11229, ϕ Ent, ϕ 1.15, Blackburn, Philipstown	[144]
2004	<i>P. fluorescens</i>	ϕ S1	[145]
2005	<i>E. coli</i> O157:H7	KH1	[146]
2005	<i>S. maltophilia</i>	C2	[147]
2006	<i>S. epidermidis</i>	456	[148]
2006	<i>S. aureus</i>	ϕ 11, ϕ 12	[149]
2007	<i>S. epidermidis</i>	K	[150]
2007	<i>E. coli</i> TG1	T7	[61]
2008	<i>S. maltophilia</i>	C2	[151]
2008	<i>P. fluorescens</i>	ϕ S1	[152]

Although the presence of polysaccharide depolymerase enzymes in phages has been reported, this characteristic is not commonly observed in most naturally isolated phages. The difficulty in isolating phages possessing EPS degrading enzymes has led to the reconstruction of phages, such as the T7 [61]. The gene-engineered T7 phage was built specifically to express a biofilm-degrading enzyme once the phage starts to infect and reproduce daughter particles inside a host. This genetic manipulation of the phage resulted in a decrease, about two orders of magnitude superior, of the bacterial biofilms when compared to the non-engineered phage.

Once the adsorption step has occurred, the phages start using the hosts' machinery to produce hundreds of new phage particles that will be released through burst of the host cell. These progeny phages can start a new cycle of host infection.

Phages are capable to kill early stage biofilms (or adhered cells). Sillankorva *et al.* (2008) reported that single cells adhered to glass surfaces of a parallel plate flow chamber

during 60 minutes and under laminar flow regime, were efficiently killed with phage ϕ S1. Cell removal was fast (20 minutes) and efficient leading to a biomass reduction of approximately 90%. Furthermore they reported that surfaces exposed to phages were impossible to be re-colonized by the bacteria [131]. Another strategy studied and proven to reduce biofilm formation by *Staphylococcus epidermidis* is the pre-treatment of catheter surfaces with phages [148].

The proximity of host cells in biofilm communities can be an advantage in biofilm treatment using phages, as the released phages stay concentrated in close proximity and therefore can start infecting a neighbouring cell much faster than in planktonic cultures where cells are not as accessible [153].

Despite the ability phages have in reducing the host cells present in biofilms, there are several factors which can influence the lytic performance of phages (ex. a change in temperature, growth media, flow, the EPS matrix, among other parameters [139, 141 - 142, 145, 150]) and lead to a decreased phage killing of their target hosts in biofilms. Also, the metabolic state of the hosts in biofilms poses a problem for phage treatment as exponentially growing cells are faster attacked than cells at the later growth phases [145, 150, 154].

Failure of phage infection of biofilms can also be caused by other factors. Doolittle *et al.* have reported that a *P. aeruginosa* phage was unable to reach the host in the deeper layers of a biofilm [140], suggesting that the phage could not penetrate through the biofilm matrix. Unsuccessful phage infection can furthermore be due to an inactivation of the phages caused by the presence of proteolytic enzymes in the biofilm matrix, however this is clearly a host dependent parameter which will reflect in different matrix components excreted.

Overall, the biofilm-phage interaction studies have demonstrated that single species biofilms can be controlled using lytic phages. Although total eradication was not observed in most of the studies reported, in general all these experiments describe a significant biomass decrease and cell number reduction [61, 137, 139, 140, 144, 148, 150, 152, 155]. Prolonged phage experiments can lead to the appearance of bacterial resistance. Tait *et al.* (2002) reported that after an extended exposure of cells to phages, the bacteria and phage started co-existing in the biofilm communities.

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1. LITERATURE REVIEW

2. ISOLATION AND CHARACTERIZATION OF PHAGES

3. PHAGE BIOFILM INTERACTION

4. PHAGE AND PLANKTONIC CELLS

5. MAIN CONCLUSIONS AND SUGGESTIONS FOR FORTHCOMING WORK

CAAAAGSAGGTAACACGCGTTCCCTTCCGATGTAATCAATCACTACT
CCATTCCAAAACTCAACATCCCGCTGACTTCCGTTCTATCTTCCGCGGCA
TAAACACCTTGATCGAAGAGTTGGGCTTGCTGGTCATGAGGTGGGCTTAA
TTCCTTCCGTGACACCTCAAGGAATGCTGACCGGATGACCTTCACTCT
AGCTTAAATCCGGGGAAGTTGCTCGAGTCTTCCACAGGGAGCAAGAAG
TACGTGCGTCAATCAATCCCTGGCGATCCCACTGAACTGAACTCCGTAC
AAAGAGTGCCCGATGTTCAAGGCCCTCCCAAGAACTGCACTCCGACTC
CTTGAAGCAGTTGCGCACACCCCGTCTGCAAGTTCAAGCTTCCCTGACCG
ACTAATCCCGTGGTTTCCTTAAGCACCCGGATCTTCACTGCAATGAGGCA
TGAAGCTCCTGAGGCCAATCGAATCCGAGATCGTTCAGGCTCCACCTGC
GCCAGCGTACCGAGTACCACCGCTCCCGCACTGCTGGCTGCCTAATTCTGTGC
CTTTCATCGCATGTGGTTCATCCCTCCCTTGACGATCCGGAAATACCACA
TCACTGACCTCCACCTAATCGGGCTCTTCATCGACCGCTCCCGCTCAACC
AATGGCGCTACCCACAAGGCATCGTCACTCAACTGAAAGAGGAAGGAGTC
CTGTAATGAAACTGTTCAAACCTCAACTTGGTACCGAGTGGGTCCCGATCT
ACCGGAAGGACCTCGACGAACTAATGCGGCTGCGCAACAGAATACGGT
GCCGCTGCTGTTCCCGGTGTCAAGCCGCACTGAACTGCTCAATCGCTGCG
EACTCAACACCTAGGECCAAGCCTGATGCACTCCCTGCACTGCAAGTA
ACCGTCACTAAGCACGCTTGCATCCGCTGCTGATGGTGCAGCCTTCCCTTC
ATCCCTCCCTAGCAGGTAGCAACGAAATCCGGCCCATATCCAGACTTACC
GTGAGCAAGCACGCTGCACTCGCTCGCGATGGTGCAGCCGATGATTGATCC
AGCGATGGAGGTAGCAACGAGAAAGGCCCATATCCAGAACTTCACGGTCA
CCAAGCACGCTCGCACTCGGCTGGCGTCTGTCAGCCGATGATTCATCCAGCG
ATCCAGGCTAGCAACGAGAAAGGCCCTGACATTATCCCAAGCACCGCTGGCA

2.1 Isolation and characterization of phage ϕ IBB-PF7A for *Pseudomonas fluorescens*

Summary

Background: *Despite the proven relevance of Pseudomonas fluorescens as a spoilage microorganism in milk related and refrigerated food products and the recognized potential of bacteriophages as sanitation agents, so far no phages specific for P. fluorescens have been closely characterized in view of their lytic efficiency. This work describes 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-PF7, was studied in detail due to its efficient lysis of a wide spectrum of P. fluorescens strains and ribotypes. Phage ϕ IBB-PF7 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.*

Conclusions: *The isolated T7-like phage, phage ϕ IBB-PF7A, due to its wide host range for different P. fluorescens strains and its fast and efficient lysis 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.*

The work presented in this sub-chapter was published in BMC Biotechnology 8:80 (27 of October 2008).

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 [1-5].

Studies with phages as control organisms of bacterial infections have greatly 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. Phages 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 food related 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, nevertheless this work is 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 it is described the isolation of phages for the food 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 broad-host range bacteriophage for a wider range of *P. fluorescens* strains. Therefore, initially several phages were isolated from a wastewater treatment plant that receives effluents from different dairy industries by enrichment with different test species of *P. fluorescens*, which had been earlier isolated from Portuguese and United States dairy plants including different ribotypes and strains with different enzymatic activities (see Table 2.1).

Table 2.1. Characteristics of dairy *Pseudomonas* spp. used. Isolate characteristics include origin, ribotypes and extracellular enzyme production [Protease (Prot), Lecithinase (Le) and Lipase (Li)].

Strain	Species	Origin	Ribotype	Prot/ Le/ Li	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]

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, Dep. of Food Science, Cornell University, Ithaca, N.Y.).

Not all *P. fluorescens* are capable of producing degradative defects in processed milk. Therefore some isolates were included in this study 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]. All NY *P. fluorescens* isolates were positive for protease, lipase, and lecithinase activity (Table 2.1), with exception of two of the strains, D3-197 and B1-020 (a potato isolate), which were able to produce only one type of enzyme, protease or lipase, respectively. Other *Pseudomonas* species unable to produce extracellular enzymes, provided by the Department of Food Science from the Cornell University, New York were also tested.

Altogether 17 phages were isolated and showed lytic activity against some of the different bacterial strains (Table 2.2). All the isolated phages were used in further screening assays using the phage spot test on bacterial lawns of the other strains in order is to characterize the host range of each phage and to select a phage with the broadest host range 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 2.2. Each group is characterized by the same lytic profile of its phage for the host strains, which either were sensitive, by showing clear or turbid plaques, or resistant (no phage plaques observed) to the distinct phage. 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; 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 did only form 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. Furthermore, the plaques of ϕ IBB-PF8 in some isolates were more turbid. Therefore, phage ϕ IBB-PF7A was selected for further characterization studies and also tested for lysis ability in other characterized *P. fluorescens* isolates provided by the Cornell University (NY).

Phage ϕ IBB-PF7A was tested with other *Pseudomonas* species and it was verified that it was not able to form plaques on *P. putida*, *P. fragi* and *P. fulva*, which are species unable to produce extracellular enzymes.

In summary, Phage ϕ IBB-PF7A was able to infect positively 5 different ribotypes of *P. fluorescens*, ribotypes 409-S-3, 422-S-2, 408-S-8, 50-S-8, and 57-S-5 respectively (Table 2.1). 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. The ribotypes 409-S-3, 408-S-8 and 422-S-2 were not classified according to the biovar to which they belong. Due to its ability to form clear large plaques on a wide host range of *P. fluorescens* strains, ϕ IBB-PF7A may be an interesting 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 (Figure 2.1).

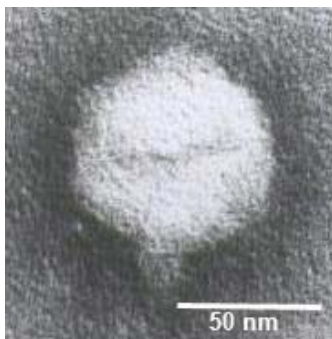


Figure 2.1. Transmission electron micrograph of *P. fluorescens* phage ϕ IBB-PF7A.

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 2.3) are similar to typical morphological values observed in members of the T7 phage group (H.-W. Ackermann, personal communication).

Table 2.2. Sensitivity of phages to different *P. fluorescens* isolates from dairy industry.

Isolate	Phage ϕ IBB-																
	PF7 A	PF7 B	PF7 C	PF7 D	PF8	PF37 B	PF33 A	PF33 B	PF33 C	PF33 D	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	-	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*
Phage group	1				2	3	4		5	6	7	8	9	10	11		

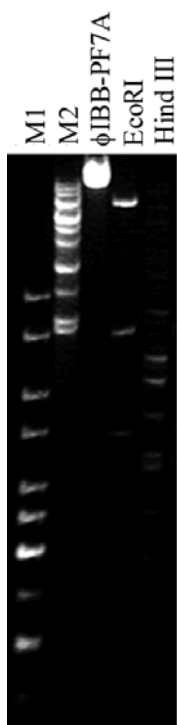
* not tested, C - clear phage plaque, T - turbid phage plaque, TT – very turbid, – no phage plaque

Table 2.3. 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	Latent period: 15 min
Halo size: 0.5 mm	Tail size: 13nm \times 8nm, tapering	Adsorption rate: 5.58×10^{-10} ml min ⁻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.

Phage DNA studies



The isolation of phage DNA and the application of restriction enzymes allows 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 (Figure 2.2). The approximate genomic size of ϕ IBB-PF7A with 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.

Figure 2.2. Restriction analysis of phage ϕ IBB-PF7A DNA. Undigested phage DNA and phage DNA digested with EcoRI and HindIII. Lanes M1 and M2: 1kb and lambda mix marker DNA ladders.

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. This 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 (Figure 2.3 and Table 2.4) 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 Figure 2.3); head-tail connector protein (p2), capsid 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.

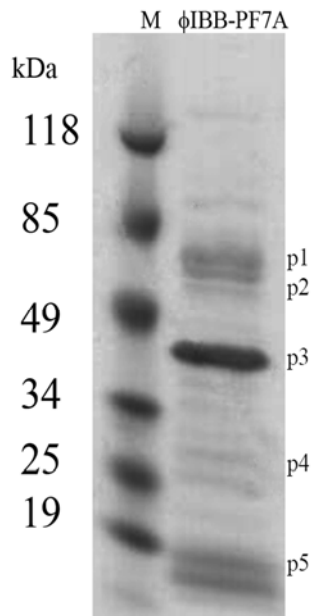


Figure 2.3. SDS-polyacrylamide gel electrophoresis analysis of phage ϕ IBB-PF7A structural proteins. Phage lysate was mixed with Laemmli buffer containing SDS, boiled for 10 min, and loaded on a 4-20% gradient gel that was electrophoresed with Tris-glycine running buffer. Lane M: molecular weight marker. p1 to p5 mark sizes of typical T7 phage structural proteins.

Table 2.4. Phage ϕ IBB-PF7A structural proteins. 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.

MW (kDa) ^a	Probable T7 protein	aa sequence	Identity (%)	BLASTp similarity (phage, 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 KEVLFG LNDS
59.7 (p2)	Head-tail con. (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) Major capsid protein 10A (phage T3, NP_523335)	AQMGGGQQIG A - - QGGQQIG ANIQQGQQIG
39.3					
38.2					
30.6 (p4)	Capsid assembly (gp9) ¹				
29.0					
25.9					
18.6 (p5)	Internal virion protein B (gp14) ¹				
16.6					

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

Phage growth characteristics

The EM, genomic and structural protein studies permit to classify ϕ IBB-PF7A as a T7-like phage. Furthermore, it was aimed 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 (Figure 2.4 and Table 2.3).

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.

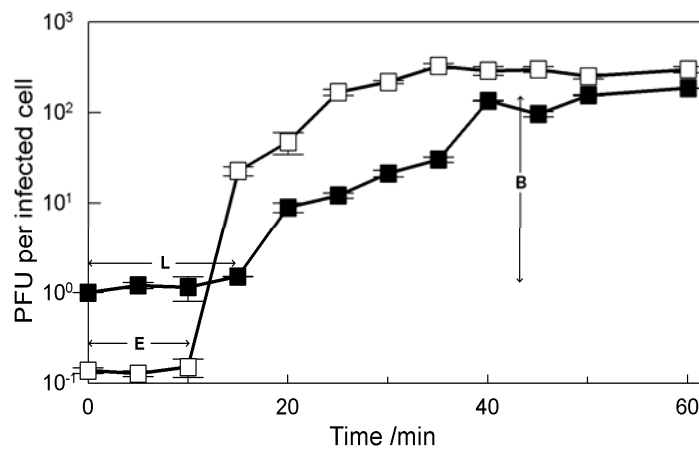


Figure 2.4. One-step growth curve of phage ϕ IBB-PF7A in *P. fluorescens* at RT. Shown are the PFU per infected cell in untreated cultures (■) and in chloroform-treated cultures (□). The phage growth parameters are indicated in the figure and correspond to: E- eclipse period; L- latent period and B- burst size.

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 (Figure 2.5).

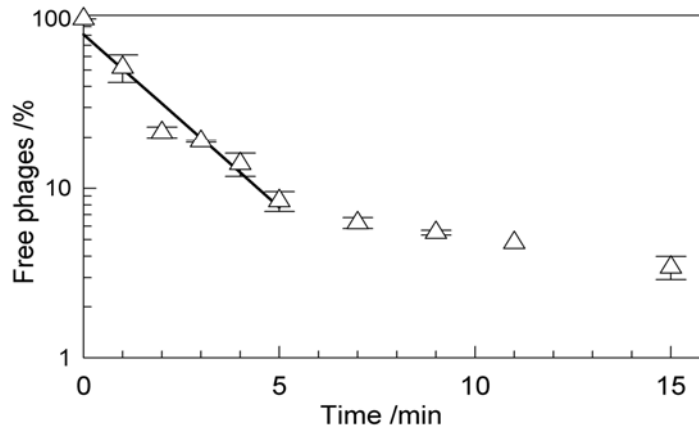


Figure 2.5. Percentage of free ϕ IBB-PF7A phages after infection of steady-state *P. fluorescens* at a MOI of 0.01.

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 (Figure 2.5). 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 2.3) 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 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 (Figure 3.2) 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 (Figure 2.5) 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].

Nowadays there are some phage products approved by different United States organizations: AgriPhage produced by Omnilytics was approved in 2005 by EPA to combat *Xanthomonas campestris* and *Pseudomonas syringae*; LISTEXTMP100 from EBI Food Safety in 2006 and LMP-102 also in 2006 from Intralytix were both approved by the FDA to combat *Listeria monocytogenes*, and BacWash from Omnilytics in the year 2006 by the USDA to target *E. coli* O157:H7 and *Salmonella*. Also, 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. plecoglossicida* 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.

Conclusions

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.

Material and Methods

Bacteria and growth conditions

The bacterial strains (see Table 2.1) 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 µl 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 ϕ IBB-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 μ l 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} $MgSO_4 \times 7 H_2O$, 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 300 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 μ l of diluted phage solution, 100 μ l 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 $\times g$ 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 (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 ($1,600 \times g$, 20 min, 4°C) the pellet was air-dried and resuspended in PBS buffer ($8 \text{ g l}^{-1} \text{ NaCl}$, $0.2 \text{ g l}^{-1} \text{ KCl}$, $0.2 \text{ g l}^{-1} \text{ KH}_2\text{PO}_4$, $1.44 \text{ g l}^{-1} \text{ Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, pH 7.5). SDS-PAGE was carried out according to Laemmli [32]. Briefly, $24 \mu\text{l}$ of sample were added to $8 \mu\text{l}$ of $4 \times$ 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 ProciseTM 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 (7,000xg, 5 min, 4°C) and resuspended in 5 ml 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.

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2.2 Complete genome sequence of the lytic *Pseudomonas fluorescens* phage ϕ IBB-PF7A

Abstract

Phage ϕ IBB-PF7A is a newly isolated T7-like bacteriophage able to infect several *Pseudomonas fluorescens* dairy isolates and to kill efficiently its host when this is found in single and dual species biofilms. These were the reasons for a further characterization of ϕ IBB-PF7A and therefore the complete genome sequence of this phage was determined. Phage ϕ IBB-PF7As' genome consists of linear double-stranded DNA with 40,973 bp. The genome has 483 bp direct terminal repeats and the GC content of this phage is 56.27 %. There are 51 open reading frames which occupy 91.78% of the entire genome and the amino acid residues range from 174 to 3996. From the total orfs, 28 have homology with T7 proteins with assigned function. There are also 23 potential putative genes with unknown function of which 7 show homology to unknown *P. putida* gh-1 phage and 2 other with hypothetical proteins of LKA1 and T7 phages. In fact, phage ϕ IBB-PF7A is closest related with phage gh-1 of *P. putida* and resulted in a homology at protein level of 55.24%. However, there is a lack of 3 class II genes in ϕ IBB-PF7A that are present in gh-1 and also this new sequenced phage has 2 DNA ligases, one of which has homology to gh-1 while the other is similar to the DNA ligase of coliphage KIF. Another striking difference between ϕ IBB-PF7A and gh-1 is the presence of the minor capsid protein 10B which is missing in gh-1. Also, there are more orfs with unassigned function in ϕ IBB-PF7A, especially before the right terminal repeat of the genome than the orfs in gh-1.

Introduction

Formerly the phylogenetic and taxonomic classification of bacteriophages (phages) was merely based on similarities in phage morphology and host range [1], phage ability to recombine, and in the similar cross-hybridization patterns with their DNA [2-4]. Today, phage genome sequencing is commonly performed for classification and characterization purposes. However, from the total estimated phage population (e.g. $> 10^{31}$ in aquatic and 10^7 in terrestrial environments), only about 5000 phages have been completely sequenced. Classification based on genome sequencing is based on the arrangement of conserved genes and the nucleotide sequence identity [5, 6]. A specific group of phages has a number of conserved genomic patterns but also a relatively high number of proteins with no match in the database which reveals the great genetic diversity existent within phages.

In the recent years, sequencing of several sequenced T7-like phages has generated a supergroup – the T7 supergroup of phages. Most members of the T7 supergroup have *Enterobacteriaceae* as hosts. Nevertheless, today there are at least 19 other phage hosts such as *Pseudomonas* [7, 8], *Yersinia* [9, 10], *Salmonella* [11, 12], *Roseobacter* [13], and *Vibrio* [14]. Some T7-like phages present more similarities with other phages and this has consequently led to the formation of subgroups within the T7 supergroup. Nevertheless, all these phages present highly conserved genome organizations differing at sequence level through the presence or absence of nonessential genes [15].

The T7 phage genome is characterized by possessing 56 genes encoding potential proteins [15]. A high percentage (45%) of the phage head volume is occupied by the genome. From the total proteins postulated, merely 35 have known function or functions. There are 25 nonessential proteins, of which only 12 are conserved across the family of T7-like phage [15].

As previously described in sub-chapter 3.1, a new phage for *P. fluorescens*, phage ϕ IBB-PF7A has been isolated from raw sewage and carefully characterized. This newly isolated phage showed morphological resemblance to T7-like phages and also presented several physico-chemical, and life cycle properties similar to the T7 phages [16]. This phage has also been the focus of several recent studies and showed to be capable of infecting a wide variety of dairy *Pseudomonas fluorescens* isolates [16], and lysing the specific host when this was found in single [17] and dual species biofilms [18]. Moreover, ϕ IBB-PF7A is the first reported phage capable to infect rod- and filamentous-shaped cells

and has a high efficiency in killing planktonic stationary cells [19]. For all these reasons, a more detailed characterization was desired and determining the genome sequence of this phage was thus the following step to further increase the knowledge on this lytic phage.

Results and Discussion

This work focuses on the determination of the complete genome sequence of the newly isolated *Pseudomonas fluorescens* phage ϕ IBB-PF7A which has been extensively used in host control studies [17-19]. Also, this phage has been classified according to several taxonomic parameters used by the International Committee on Phage Taxonomy including host range determination, TEM visualization and life cycle studies [16].

ϕ IBB-PF7A nucleotide sequence and general sequence properties

In a previous characterization work, phage ϕ IBB-PF7A showed to resemble morphologically to T7-like phages. Here, the DNA sequence of phage ϕ IBB-PF7A for *P. fluorescens* was determined. The genome of ϕ IBB-PF7A consists of linear double-stranded DNA with 40,973 bp and this size is among the range of other T7-like phages where the smallest reported is the *P. putida* phage gh-1 (37,359 bp) [7] and the largest is the 45,874 bp *Vibrio parahaemolytic* phage VpV262 [14]. The direct terminal repeats (DTR) of ϕ IBB-PF7A are 483 bp, which are longer than the DTRs of all other members of T7, which reportedly range from 160 bp in T7 [20] to 428 bp in *P. aeruginosa* phage LKD16 [21]. Phage ϕ IBB-PF7A has an overall genomic guanine plus cytosine (GC) content of 56.27 % which is in the range of the GC content observed in T7 members (46.2 – 62.3%) [8].

ϕ IBB-PF7A gene organization

Phage ϕ IBB-PF7A was examined for open reading frames (orf) of 150 bp or longer size using the GeneMark software and visual inspection was also used to scan the complete genome sequence. The search resulted in 51 predicted putative genes or orfs which occupy 91.78 % of the entire nucleotide genome. It is believed that phages with DNA molecules whose length is limited by a virion of fixed size tend to pack the maximum amount of useful information efficiently into the DNA molecule (20).

In phage ϕ IBB-PF7A, the orfs length range from 174 to 3996 amino acid residues. The predicted putative genes were blasted and compared with the non-redundant protein database and the results are described in Table 2.5. From these 51 orfs, 28 have homology with T7 phage proteins with known function. Therefore, the T7 gene nomenclature, where genes are numbered sequentially in the order that they are transcribed, was used for

assigning the genes of ϕ IBB-PF7A phage. From the total predicted putative genes, 49 are transcribed from the same strand and only two genes, orfs 14 and 18 respectively, are transcribed from the reverse strand (Table 2.5 and Figures 2.8 and 2.9).

Orfs which showed no similarities to known putative proteins in the database or gave homology to unknown or hypothetical proteins of other phages were numbered from 1 to 23 (orf1 to orf23), starting from the left end of the genome sequence. Seven orfs with unknown function had some similarity with orfs of phage gh-1, 1 orf with a hypothetical protein of phage LKA1 and 1 orf with a hypothetical protein of phage T7, respectively. These 23 predicted putative genes of unknown function are probably nonessential orfs or regulatory signals. Nevertheless, no protein coding function can be speculated without more detailed studies. The initiation codon for 46 of the 51 potential genes is ATG while 3 orfs have GTG and 2 orfs have TTA as the initiation codon. Like in all other T7 supergroup members, the temporal and functional distribution of phage ϕ IBB-PF7A genes is tightly organized and all genes are packed close to each others. The genome organization and gene order of phage ϕ IBB-PF7A seems largely conserved in gh-1 although there are some differences. The average protein homology percentage obtained between the genes of these two *Pseudomonas* phages was 55.42% (Table 2.5).

At the nucleotide level, there are regions that are identical (red line) but also several parts where the straight line is interrupted. The first big gap in the straight line occurs immediately after RNA polymerase and before the primase/helicase and the highest dissimilarity is observed in the region corresponding to the internal virion proteins (gp12 to gp15) (Figure 2.6). Thus, although there is similarity at protein level the homology percentage at nucleotide level is decreased.

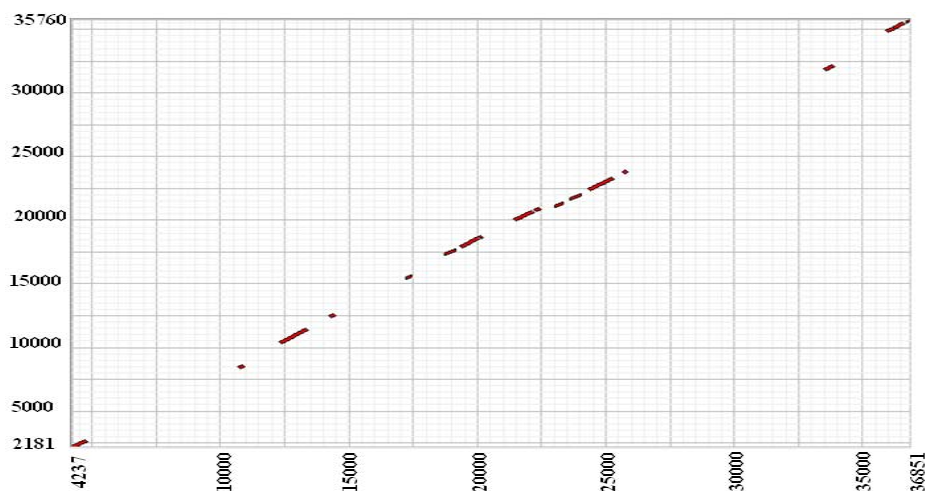


Figure 2.6 – Dot blot comparison of ϕ IBB-PF7A and gh-1 genomes. Dot plot analysis was performed with the Genome Comparison Viewer (www.softberry.com) with the genomic DNA sequence of ϕ IBB-PF7A in x axis and gh-1 in y axis.

Adsorption and host range

Phage adsorption to a host occurs via interaction of the tail fibers with molecules present on the host surface. The ability of phages to interact and adapt to a new host is due the rather malleable structure of the tail fibers that can easily adapt to a variety of receptors in order to start adsorption [15]. The phage studied here, ϕ IBB-PF7A, is the first T7-like phage for *P. fluorescens* for which the genome has been sequenced. This broadens the host members of the T7 supergroup of phages to one more bacteria – *P. fluorescens*.

DNA penetration

After phage adsorption to a host and the phage DNA is injected to the hosts cytoplasm. Some genes are known to carry this function and in phage ϕ IBB-PF7A all these necessary genes are present, namely the gp14, gp15 and gp16. These gene products when combined form a channel right from the tail tips all the way through the cell envelope allowing the penetration of the DNA. This penetration or internalization takes usually place in 10 minutes and the genomes are internalized by transcription. This 10 minute period is quite long but it is due to the regulation that occurs by the insertion of the genes in the proper manner: first the early genes, followed by middle and lastly the late genes.

φIBB-PF7A gene arrangement

Phage φIBB-PF7A has a gene clustering analogous to T7 and therefore a similar gene organization was suggested. Genes were divided in three clusters – class I, II and III genes respectively. As Blastn grouped φIBB-PF7A with the *P. putida* phage gh-1 (Figure 2.7) the gene clustering of these 2 phages was compared. The cluster I groups all early genes which are responsible for the transition to phage metabolism (Figure 2.8). Cluster II has the so called middle genes which are genes involved in DNA metabolism or phage replication (Figure 2.9), while class III contains the late genes that are all structural proteins, genes responsible for the virion assembly, packaging and maturation proteins and also the genes which will lyse the hosts (Figure 2.10).

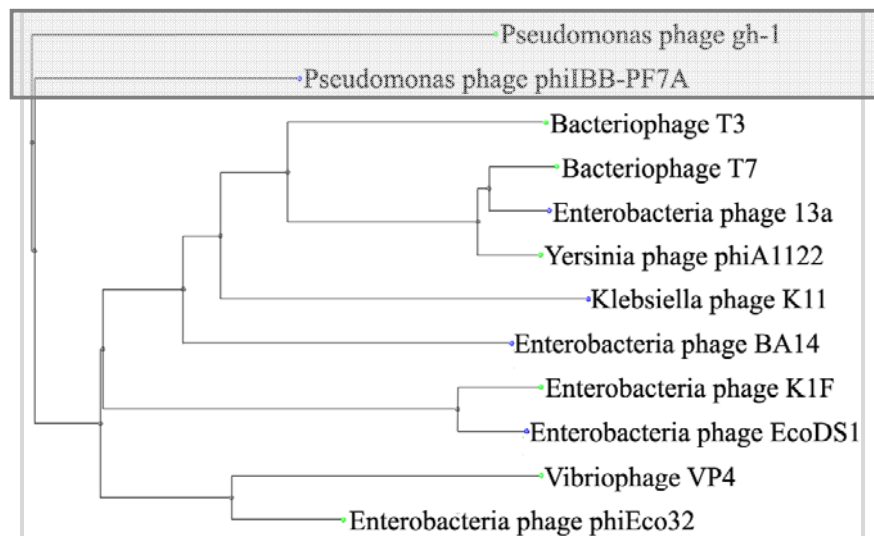


Figure 2.7 – Phylogenetic relation between phage φIBB-PF7A and other T7-members. The distance tree of results was generated using blastn.

Table 2.5 – Features of phage ϕ IBB-PF7A open reading frames and their homologies to other T7-like phages.

Gene	Strand	Left End	Right End	Gene Length	Start codon	Stop codon	MW (kDa)	PI	GC content (%)	Similarity	Best homolog	Identity (%)	BLAST score	E value	Accession no.
orf1	+	252	602	351	ATG	TAA	108.49	5.21	58.90	unknown					
orf2	+	602	928	327	ATG	TAA	101.37	5.25	55.35	unknown					
orf3	+	996	1511	516	ATG	TGA	159.77	5.16	57.36	unknown					
orf4	+	1508	2215	708	ATG	TAA	218.78	5.09	57.34	unknown					
orf5	+	2217	2594	378	ATG	TGA	116.60	5.19	59.79	unknown					
orf6	+	2591	2989	399	ATG	TGA	126.70	5.21	56.89	unknown					
gp1	+	3123	5798	2676	GTG	TAA	827.13	4.82	56.02	polymerase	gh-1	63	1102	0.0	NP_813747
orf7	+	5808	6002	195	ATG	TAA	60.16	5.31	54.36	unknown					
orf8	+	6067	6297	231	ATG	TGA	71.21	5.27	59.74	unknown					
orf9	+	6294	6572	279	GTG	TAA	85.98	5.24	56.27	unknown					
gp1.3	+	6573	7100	528	ATG	TGA	163.36	5.15	58.33	DNA ligase	gh-1	47	134	3e-30	NP_813751
gp1.3	+	7054	7557	504	ATG	TGA	156.06	5.16	57.54	DNA ligase	K1F	55	174	2e-42	YP_338096
orf10	+	7636	8100	465	ATG	TAA	143.66	5.17	55.70	unknown					
orf11	+	8324	8989	666	ATG	TGA	205.74	5.10	57.81	unknown	gh-1	45	188	3e-46	NP_813752
orf12	+	9120	9461	342	ATG	TAA	105.43	5.21	57.89	unknown	gh-1	51	102	9e-21	NP_813755
gp2.5	+	9537	10232	696	ATG	TAA	215.29	5.10	56.90	ssDNA binding	T3	52	207	5e-52	NP_523311
gp3	+	10233	10670	438	ATG	TAG	135.52	5.18	56.85	endonuclease	gh-1	61	170	4e-41	NP_813757
gp3.5	+	10683	11144	462	ATG	TAA	142.80	5.17	56.49	lysozyme/amidas e	gh-1	60	184	1e-45	NP_813758
orf13	+	11210	11773	564	ATG	TGA	174.20	5.14	55.50	unknown	gh-1	40	108	3e-22	NP_813759
gp4	+	12036	13475	1440	ATG	TAA	446.20	4.97	55.42	primase/helicase	gh-1	72	707	0.0	NP_813761
orf14	-	13472	13765	294	TTA	CAT	90.48	5.08	55.44	unknown	gh-1	44	110	5e-04	NP_813762
orf15	+	13755	14198	444	ATG	TGA	137.14	5.19	54.28	unknown	gh-1	36	121	2e-05	NP_813763
gp5	+	14185	16323	2139	ATG	TAA	662.43	4.87	57.18	DNA polymerase	gh-1	64	904	0.0	NP_813764

Table 2.5 (Cont.) – Features of phage ϕ IBB-PF7A open reading frames and their homologies to other T7-like phages.

Gene	Strand	Left End	Right End	Gene Length	Start codon	Stop codon	MW (kDa)	PI	GC content (%)	Similarity	Best homolog	Identity (%)	BLAST score	E value	Accession no.
orf16	+	16325	16639	315	ATG	TGA	97.69	5.26	54.92	unknown	gh-1	33	43.1	7e-03	NP_81376_5
gp5.7	+	16642	16851	210	ATG	TGA	64.87	5.30	57.62	gp5.7	gh-1	59	88.2	2e-16	NP_81376_6
gp6	+	16848	17762	915	ATG	TAA	283.74	5.05	57.81	exonuclease	gh-1	59	323	1e-86	NP_81376_7
gp6.5	+	17870	18136	267	ATG	TAA	82.49	5.28	50.94	gp6.5	gh-1	60	63.2	6e-09	NP_81376_8
gp6.7	+	18123	18413	291	ATG	TAG	90.16	5.27	53.61	virion protein essential	gh-1	50	68.6	1e-10	NP_81376_9
gp7.3	+	18423	18743	321	ATG	TGA	99.71	5.26	56.07	virion protein head-tail	gh-1	55	43.5	5e-03	NP_81377_1
gp8	+	18756	20363	1608	ATG	TAA	497.47	4.93	55.66	connector capsid	gh-1	77	813	0.0	NP_81377_2
gp9	+	20429	21343	915	ATG	TAA	282.87	5.04	57.81	assembly major	gh-1	49	256	2e-66	NP_81377_3
gp10A	+	21432	22457	1026	ATG	TAA	316.91	5.02	56.14	capsid 10A	gh-1	79	474	4e-132	NP_81377_4
gp10B	+	22499	22987	489	ATG	TAA	151.57	5.18	54.81	minor capsid 10B	phiYe03-12	58	66.6	6e-10	NP_05210_8
gp11	+	23052	23639	588	ATG	TAA	181.68	5.13	55.10	tail tubular A	gh-1	72	291	2e-77	NP_81377_5
gp12	+	23650	26073	2424	ATG	TAA	749.42	4.84	57.63	tail tubular B	gh-1	65	1050	0.0	NP_81377_6
gp13	+	26102	26539	438	ATG	TAG	135.34	5.18	55.94	Internal virion A	gh-1	45	105	1e-21	NP_81377_7

Table 2.5 (Cont.) – Features of phage ϕ IBB-PF7A open reading frames and their homologies to other T7-like phages.

Gene	Strand	Left End	Right End	Gene Length	Start codon	Stop codon	MW (kDa)	PI	GC content (%)	Similarity	Best homolog	Identity (%)	BLAST score	E value	Accession no.
gp14	+	26572	27108	537	ATG	TAG	166.27	5.14	58.29	Internal virion B	gh-1	47	125	1e-27	NP_813778
gp15	+	27117	29330	2214	ATG	TGA	684.69	4.86	56.55	Internal virion C	gh-1	42	589	3e-166	NP_813779
gp16	+	29334	33329	3996	ATG	GTA	7	4.73	58.00	Internal virion D	gh-1	46	1139	0.0	NP_813780
gp17	+	33392	35029	1638	ATG	TAA	504.71	4.90	57.94	tail fiber	gh-1	55	256	3e-66	NP_813781
gp17.5	+	35039	35242	204	ATG	TAA	63.17	5.33	51.96	Lysis protein	K11	39	49.7	7e-05	YP_418245
gp18	+	35235	35492	258	ATG	TGA	79.46	5.27	54.65	DNA packaging A	gh-1	52	77.8	2e-13	NP_813784
gp18.5	+	35492	35929	438	ATG	TGA	135.5	5.19	54.34	cell lysis protein	gh-1	34	73.9	3e-12	NP_813785
gp19	+	35926	36264	339	GTG	TGA	104.5	5.22	53.69	DNA packaging B	gh-1	83	154	3e-39	NP_813786
gp19	+	36366	37691	1326	ATG	TAA	410.4	4.97	56.56	DNA packaging B	gh-1	73	658	0.0	NP_813786
orf17	+	37912	38085	174	ATG	TGA	53.69	5.34	54.60	hypothetical protein	T7	47	46.2	8e-04	ABR22985
orf18	-	39145	39348	204	TTA	CAT	62.97	5.12	47.06	unknown					
orf19	+	39347	39838	492	ATG	TGA	151.7	5.15	55.89	hypothetical protein	LKA1	39	88.6	1e-16	YP_001522848
orf20	+	39835	40104	270	ATG	TGA	1	5.28	55.19	unknown					
orf21	+	40239	40589	351	ATG	TAA	83.65	5.21	58.97	unknown					
orf22	+	40589	40915	327	ATG	TAG	108.4	5.25	55.35	unknown					
							101.3								

Early gene expression

The genome of phage ϕ IBB-PF7A has after the 483 bp left DTR the class I genes (Figure 2.8) which are synthesized about 2 to 8 minutes pos-infection. The host RNA polymerase transcribes these class I genes which include genes with functions to overcome host restriction and to convert the metabolism of the host cell to the production of phage proteins, e.g., synthesis of phage promoter-specific RNA polymerase (gp1). Unlike in *P. putida* phage gh-1, which has no putative genes before gp1, phage ϕ IBB-PF7A has 6 orfs before gp1. However, none of these 6 genes presents similarities with genes known in the database. T7 phages have several different genes before gp1 (gp0.3 to gp0.7). These new orfs present in ϕ IBB-PF7A, before gp1, may have a specific function in the phage infection cycle that is similar to the functions of the known early T7 genes. Nevertheless, the lack of similarity is intriguing. Also, the genome of phage ϕ IBB-PF7A presents two genes with homology to DNA ligase (gp1.3): one which is 528 bp and resembles to the gene of phage gh-1 and the other, 504 bp long, shows similarity to the gp1.3 of coliphage K1F (Table 2.5).

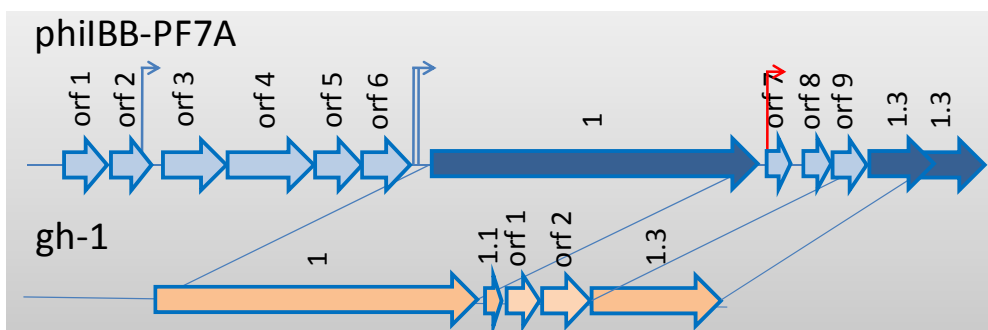


Figure 2.8 – Putative distribution of early or class I genes in *P. fluorescens* phage ϕ IBB-PF7A and comparison with *P. putida* phage gh-1. Unknown orfs are in lighter colors than genes with assigned function.

Middle gene expression

The class II genes are transcribed by the phage RNA polymerase (gp1) and they are synthesized from about 6 to 15 minutes pos-infection. All the first T7 class II genes (gp1.4, gp1.5, gp1.6, gp1.7 and gp1.8) are missing in the genome of phage ϕ IBB-PF7A (Figure 3.9). Four of these five first class II genes are nonessential and only the gp1.7 is considered an essential gene. Gp1.7 is reportedly a full-length non conserved gene that is beneficial for phage growth [15].

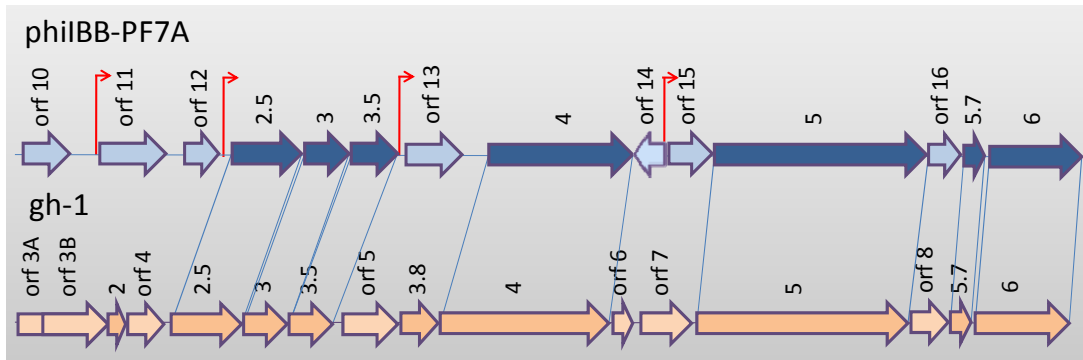


Figure 2.9 – Putative gene distribution of middle or class II genes in *P. fluorescens* phage ϕ IBB-PF7A and the comparison with *P. putida* phage gh-1. Unknown orfs are in lighter colours than genes with assigned function.

Besides the lack of all these first class II genes, the genome of ϕ IBB-PF7A lacks also other genes such as: the T7 inhibitor of host RNA polymerase (gp2), the nonessential homing endonuclease (gp3.8), nonessential gp4.3 and nonessential gp5.5 as well as the genes gp3.2 and gp3.7. Also, the phages ϕ IBB-PF7A and gh-1 have both 7 orfs with unknown function that, although not presenting protein homology with T7, might assume identical functions to some class II T7 phage genes.

Late gene expression

Like class II genes, the late genes or class III genes are also transcribed by the phage RNA polymerase. Like in all T7 members, in phage ϕ IBB-PF7A the class III genes start with exonuclease (gp6). In fact, the majority of T7 putative genes are present in ϕ IBB-PF7A (Figure 2.10). However, the genome of ϕ IBB-PF7A doesn't possess the nonessential protein gp7 which is usually involved in the recognition of the host range [15]. Additionally, there are 6 orfs all situated just before the right DTR. The major striking difference in class III genes between ϕ IBB-PF7A and phages gh-1 is the presence of the minor capsid protein 10B (gp10B). In the capsid shell of T7 coliphages there are 415 molecules of capsid protein (gp10). Of these 415 molecules, 95% are of major capsid protein 10A (gp10A) and only a small percentage of gp10B [15]. The gp10B of phage ϕ IBB-PF7A showed 58% protein homology with the gp10B of *Yersinia enterocolitica* phage ϕ Ye03-12 and also 57% with coliphage T3.

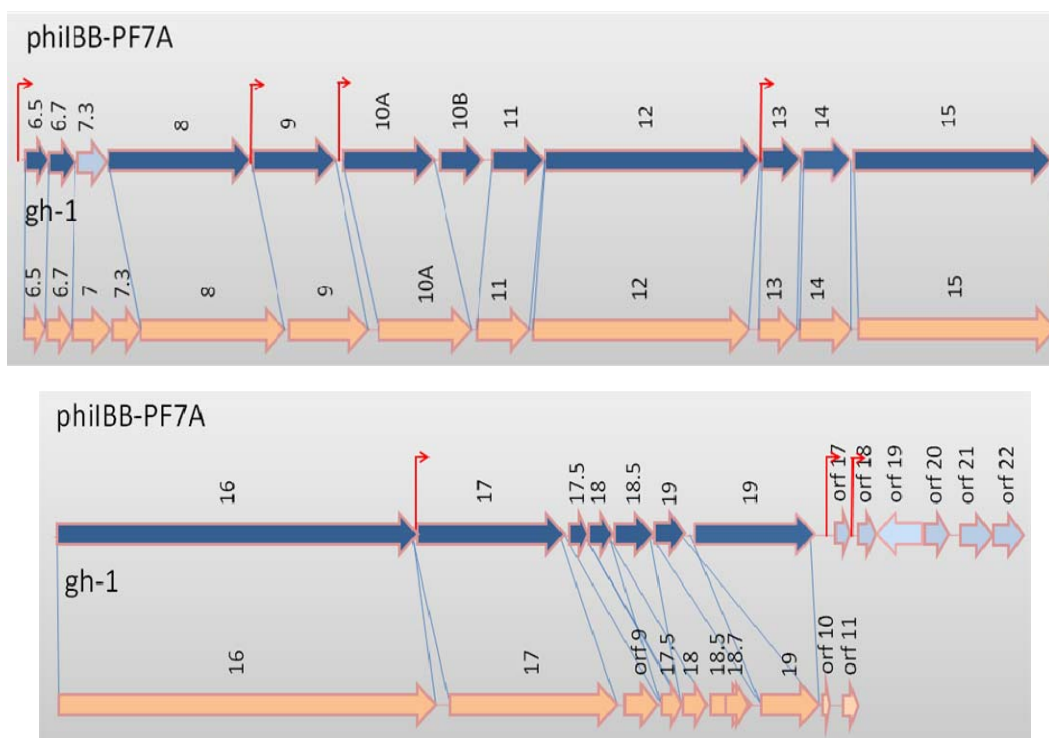


Figure 2.10 – Putative gene distribution of late or class III genes in *P. fluorescens* phage ϕ IBB-PF7A and the comparison with *P. putida* phage gh-1. Unknown orfs are in lighter colours than genes with assigned function.

Phage ϕ IBB-PF7A possesses the gene internal virion protein D (gp16). Gp16 presents peptidoglycan hydrolase activity which is essential for enlarging a hole across the cell wall of stationary cells or the cell walls when hosts were infected at low temperatures [15]. In previous work, this phage has shown to be able to infect efficiently stationary cells and cells grown below the optimal temperature [17, 19].

Putative regulatory elements

No tRNA genes were predicted in the ϕ IBB-PF7A genome using tRNAscan-SE. Also, no transmembrane helices were found using TMPred and TMHMM. Although no tRNA genes were identified in the sequence of ϕ IBB-PF7A, several potential putative transcription promoters were identified in the DNA sequence using PHIRE (Table 2.6).

Table 2.6 – Predicted promoter sequences in phage ϕ IBB-PF7A using PHIRE and degenerate consensus sequence are presented.

Name	Promoter sequence	Number of mismatches	Transcription	
			Beginning	End
A3	CACAGTGACATCGGGCTGGATGCGTCCAGTAACCTCAACGCTTTGACCGA		875	920
C	CTTAACTTAAAGACCCCTTTAAGATCCTTTAAGATTACTCTTATAGTAAT		3033	3078
C	GACCCTTTAAGATCCTTTAAGATTACTCTTATAGTAATTATCATTAAGTA		3045	3090
ϕ orf8	CCCCTACCAAAACAGGGTG	3	06005	06024
ϕ orf11	ACCCTACCAAAACAGGGAC	2	08263	08282
ϕ 2.5	ACCCTACCAGAACAGGGAG	2	09464	09483
ϕ orf13	ACCCTACCAGAACAGGGAC	3	11147	11166
ϕ orf15	ACCCTACCTAAACAGCTTG	3	13961	13980
ϕ 6.5	CCCCTACCTAAACAGGGAG	1	17798	17817
ϕ 9	ACCCTACCTAAACAGGGAG	0	20366	20385
ϕ 10A	ACCCTACCTAAACAGGGAG	0	21359	21378
ϕ 13	ACACTCACCACAACAGGGAG	3	26076	26095
ϕ 17	CCCCTACCTAAACAGGGAG	1	33333	33352
ϕ orf17	ACCCTACCTAAAGAGGGAC	2	37734	37753
ϕ orf18	ACCCTACCTAAACAGGGAT	1	38714	38733
Degenerate consensus	-10 -5 +1 +5 ACCTCACTCTAAACAGGAG			
gh-1	ACCTCACTRTGGCHSCM			
T7	CGACTCACTATAGGGAGA			

The phage RNA polymerase (RNAP) in all T7 group phages is responsible for the recognition of phage-specific promoters and these promoter sequences are found in all the members of the T7 group for which genome data is available. Like in T7 phage, there were identified 3 early host RNAP promoters near the left end of phage ϕ IBB-PF7A (blue lines with arrowhead in Figure 2.8). These promoters are necessary for the expression of the RNA polymerase. The first one identified is possibly the promoter A3 found in T7 phages. The second and third promoters are possibly the same ones, as they are closely located, and may correspond to the minor *E. coli* promoter C.

T7 group phages code for an RNAP with a strict template specificity for its own genome. In phage ϕ IBB-PF7A, there were 12 phage RNAP specific promoters recognized (Table 2.6) and they were named according to the gene right after they appear. The promoters in ϕ IBB-PF7A correspond to the red lines above the genome (Figures 2.9 and 2.10). These promoter sequences have some mismatches (see Table 2.6) and a degenerate consensus sequence, ACCCTCACCTAAACAGGGAG, was derived based on all sequences. 12 of the 13 putative promoters lie in intergenic regions and only 1 is located approximately 200 bp from the beginning of orf 15. Nevertheless, all the other 11 sites are excellent promoter candidates. In T7 and gh-1 phages there are 16 promoters. The specificity of the RNAP is determined by the positions -10 and -11 and it is the position -2 that establishes the promoter strength (23). The highest relative utilization of the promoter is observed when there is a T at the -2 position. Unlike in phage gh-1 and T7, both of which have T at -2, ϕ IBB-PF7A has A and therefore a lower promoter utilization. Nonetheless, the promoter positions -10 and -11 are identical to the consensus sequence of gh-1 (C and C, respectively) with the exception of position -10 of promoter ϕ 13. The promoter positions -10 and -11 of ϕ IBB-PF7A are distinct from the T7 promoter positions which are G and A, respectively. Phage ϕ IBB-PF7A has 6 promoters that are also identified in gh-1 and T7 which are: ϕ 2.5, ϕ 6.5, ϕ 9, ϕ 10A, ϕ 13 and ϕ 17, respectively. There are also promoters that lie just before orfs of unknown function (Table 2.6 and Figure 2.10). The promoter sequences which are found in analogous genomic positions to the ones in gh-1 and T7 suggest that the regulation of phage mRNA synthesis is also well conserved within the group. Furthermore, the consensus sequences of gh-1 and T7 are 50 and 39% dissimilar over the 18 nucleotides compared.

FindTerm program (www.softberry.com) was used to search for transcription terminators. T7 and gh-1 phages have 3 terminators identified. However, FindTerm only

identified one putative major terminator. This terminator is positioned at 22994 to 23040 which is just downstream of gene 10. This suggests the presence of the T \emptyset terminator as the location is equivalent to the one found in gh-1 (named T_{Late1} in this phage) and also in T7 phages.

Lysis genes

Endolysin is a soluble muralytic enzyme produced by dsDNA phages. Like in T7 phages, gp3.5 of ϕ IBB-PF7A is proposed to be the endolysin which possess N-acetylmuramoyl-L-alanine amidase activity. The access of endolysins to the cell wall occurs through the presence of a secondary lysis factor known as holin. The small membrane protein gp17.5 of ϕ IBB-PF7A is proposed as holin. Phage ϕ IBB-PF7A has also one more lysis gene which is the 18.5. However, unlike T7 phages, the genome of ϕ IBB-PF7A is lacking the host cell lysis gene 18.7.

Summarizing, the genome sequencing of ϕ IBB-PF7A showed similarity to T7 phages and allowed the formation of a new sub-group within the T7 supergroup. So far, this new sub-group consists of only phages ϕ IBB-PF7A and gh-1.

Materials and Methods

Bacteria and bacteriophage ϕ IBB-PF7A

The bacteria used for phage propagation was isolated from a dairy industry and the bacteriophage was isolated from raw sewage. Bacteria was grown at 30 °C in Tryptic Soy Broth (TSB, Fluka) and glycerol stocks were done and stored frozen at -80.

Bacteriophage ϕ IBB-PF7A propagation

For phage amplification, the plate lysis and elution method described by Sambrook & Russel [22] was used with some modifications. Briefly, 1 ml of phage (1×10^3 PFU ml⁻¹) and 1 ml of overnight grown host were mixed with 30 ml of TSB soft agar (TSB + 0.6% agar) and added to 250 ml T-flasks with a thin layer of TSA media. The soft-agar layer was allowed to solidify and the flasks were incubated at 30 °C for 7 h. Afterwards, the flasks were eluted with SM buffer and the phage lysate was first concentrated with PEG8000 and then purified with chloroform. Samples in SM buffer were stored at 4 °C until further use.

DNA isolation

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

Phage DNA sequencing

Phage ϕ IBB-PF7A DNA was sheared with a nebulizer and sizes between 1 and 4 kb were cut from a 1% 1×TAE agarose gels after overnight electrophoresis at 30V. Afterwards, the fractions were purified, end repaired and ligated overnight at 16°C into pZero cloning vector (Zero background/Kan cloning kit, Invitrogen) which had been previously digested with EcoRV. After overnight ligation, chemical transformation to One Shot® TOP10 cells (Invitrogen) was performed. The cells were recovered in SOC medium, incubated for 1 h at 37°C and 225 rpm (Titramax plate shaker, Heidolph) and afterwards plated onto a LB-Kan containing X-Gal plate. Selected clones were picked with a colony picker (QPix2) and grown overnight in LB medium at 37 °C and 225 rpm. The cultures were centrifuged and plasmid DNA was extracted and purified and eluted in $0.1 \times$ TE buffer. PCR was performed with T7, SP6 primers or with oligos designed

throughout the sequencing experiments. For sequencing, Big Dye chemistry was used and sequencing was carried out in ABI 3700 DNA Sequencer (Applied Biosystems). Sequence reads were assembled using GAP4 (Staden package software). Sequences were assembled into contigs, and gaps were linked through a primer-walking technique with 20-mer oligonucleotide primers and pure ϕ IBB-PF7A DNA until all contigs assembled into a single sequence.

ORF prediction and annotation

Prediction of open reading frames (orfs) was performed using GeneMark.hmm, OrfFinder and by visual inspection of the sequence searching ATG as start codons. For tRNA gene identification, the tRNAscan-SE program was used. Translated orfs were compared with known protein sequences using Blastx and Blastn, using the non-redundant public GenBank database.

Molecular masses and isoelectric points of phage ϕ IBB-PF7A translated putative proteins were determined with ExPasy. Promoter predictions were calculated using promoter predictor (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl).

A search for transmembrane helices was done by using the TMHMM program.

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2.3 Isolation and characterization of *Staphylococcus lentus* phage ϕ IBB-SL58B

Abstract

*This work describes the isolation and characterization of a *Staphylococcus lentus* phage capable to infect also *S. sciuri* and *S. simulans*. The phage was characterized morphologically by TEM and the physico-chemical properties were studied. Phage ϕ IBB-SL58B belongs to the Podoviridae family but could not be assigned to any group of phages as the virions possess uncharacteristic morphology, as evaluated by TEM. Furthermore, phage ϕ IBB-SL58B growth parameters differ from the typical values known for Podoviridae genera. Phage ϕ IBB-SL58B presents 9 distinctive structural proteins of which the major component is a 47.8 kDa polypeptide. The protein bands excised and analyzed by N-terminal sequencing showed no homology to known structural proteins. The genome of phage ϕ IBB-SL58B proved to be resistant to cleavage by a number of restriction enzymes (*Eco* RI, *Eco* RV, *Hind* III, *Pst* 509I, *Bam*HI, *Sau* 3AI, *Sma* I, *Spe* I, *Sph* I, and *Xba* I). Moreover, the DNA sequencing showed that there are genes which resemble to T7 like phages however the identity percentage is minimal. Thus, all these results suggest that phage ϕ IBB-SL58B is clearly a novel phage and the first phage of the Podoviridae family that has a gram-positive host.*

Introduction

Staphylococci are divided into coagulase positive (CoPS) and negative (CoNS) strains. *S. aureus* is the most known pathogenic CoPS and is frequently isolated from clinical environments while among the best studied CoNS is *S. epidermidis*. Although CoNS are widespread in nature and considered commensal inhabitants of humans and animals' skin, mucous membranes and other body sites (Koneman *et al.*, 1988; Quinn *et al.*, 1994), there has been an increase of clinical infection due to these strains. CoNS are also frequently associated with animal diseases and especially isolated from infected bovine and caprine mammary glands (Bedidi-Madani *et al.*, 1998; Birgerrson *et al.*, 1992; Lilenbaum *et al.*, 1987; Devriese, 1990; Devriese *et al.*, 1985; Kloos, 1980; Nagase *et al.*, 2002). The increase of CoNS infection is due to the high resistance to antibiotics (Otto, 2004).

Staphylococcus members belong to different groups. The CoNS *S. sciuri* group consists of the species *S. sciuri*, *S. lentus*, *S. vitulinus*, and *S. pulvereri* (Kwok and Chow, 2003) and besides animals skin and mucosal surfaces, these staphylococci have been isolated from different foods of animal origin (Garcia *et al.*, 2002; Papamanoli *et al.*, 2003), from soil, sand, water (Kloos, 1980; Oger *et al.*, 2003) as well as from hospital environments (Dakic *et al.*, 2005). The members are novobiocin-resistant, oxidase positive and rarely considered pathogenic for humans. Nevertheless, there has been an increase of different infections due to these bacteria (Dakic *et al.*, 2005; Stepanovic *et al.*, 2005) and nowadays some CoNS have shown to be opportunistic human pathogens.

This work describes the morphological, physico-chemical and genetical characterization of a *Staphylococcus lentus* phage which has been recently isolated.

Results and Discussion

Selection of the *Staphylococcus* lytic phage candidate

Staphylococcus spp. phages, were isolated from raw sewage for 10 different dairy isolates belonging to *S. sciuri*, *S. lentus* and *S. simulans* (Table 2.7). Phages were found for only four of the host isolates investigated, namely the *S. lentus* isolates 57 and 58 and for *S. sciuri* isolates 46 and 51. Also, there was not found any phage for *S. simulans*.

Table 2.7 – Bacterial species and strains used and phages isolated for the different hosts.

Bacterial species and strain designations	Phages designations for the isolated hosts
<i>Staphylococcus lentus</i> : 57, 58	<i>Staphylococcus lentus</i> : φIBB-SL57A, φIBB-SL57B, φIBB-SL57C, φIBB-SL57D, φIBB-SL58A, φIBB-SL58B
<i>Staphylococcus sciuri</i> : 45, 46, 50, 51, 55, 56, LC4	<i>Staphylococcus sciuri</i> : φIBB-SC46, φIBB-SC51
<i>Staphylococcus simulans</i> : 53	<i>Staphylococcus simulans</i> : -

The isolated phages (Table 2.7) were further investigated in screening assays, where all phages were tested against all bacterial isolates (Table 2.8), in order to select the most effective lytic phage that would be further characterized.

Table 2.8 – Screening of phages for *Staphylococcus* spp. for selection of the most efficient phages.

Isolate	φIBB-							
	SL57A	SL57B	SL57C	SL57D	SL58A	SL58B	SC46	SC51
45	-	-	T	T	T	T	T	-
56	-	-	T	T	T	T	-	-
55	-	-	T	T	T	T	-	-
53	-	T	T	T	T	T	-	-
46	-	T	T	T	T	T	T	-
51	T	T	T	T	T	T	T	T
50	T	T	T	T	T	T	-	-
57	T	T	T	T	T	T	-	-
58	C	C	C	C	C	C	-	C
LC4	C	C	C	C	C	C	-	C

C - clear phage plaque; T - turbid phage plaque; – no phage plaque

Most of the phages isolated from raw sewage produced turbid plaques in the different hosts. From the list of isolated phages, φIBB-SL57C, φIBB-SL57D, φIBB-SL58A and

φIBB-SL57B gave similar screening results and were all capable to infect *S. lentus*, *S. simulans* and *S. sciuri*. This suggests that they possibly belong to the same phage species or are in fact the same phage. All of them showed a very small plaque (1.5-2 mm) and a considerably big halo (2-2.5 mm) on the bacterial lawn of isolate 58 and an even smaller plaque size on isolate LC24. Although phage φIBB-SL57C and φIBB-SL57D formed a turbid plaque on the isolation host (isolate 57), these phages formed clear plaques on the lawns of isolate 58. Thus, phages isolated for *S.lentus* nr. 58 were preferred over the phages for *S. lentus* nr. 57. Phage φIBB-SL58B, was selected, rather randomly, for further characterization studies. This study shows that, the majority of the isolated phages, for dairy *Staphylococcus* spp., can be considered polyvalent.

Morphology of the phage particles

Morphological characterization of phage φIBB-SL58B using Transmission Electron Microscopy (TEM) was performed (Figure 2.11) showed that this phage belongs to the family of *Podoviridae* and as it has a short tail it also belongs to type C in Bradley's classification (Bradley, 1967). The *Podoviridae* family consists of different genera: T7-like, P22-like, φ29-like, N4-like and unassigned viruses and although there are some morphological similarities between phage φIBB-SL58B and group N4-type phages, the definite morphological assignment of phage φIBB-SL58B can not be made due to the uncharacteristic morphology of the virion particles (Figure 2.11) (Dr. H. W. Ackermann, personal communication). These particles have head and tail sizes of approximately 64nm and 13 × 9 nm, respectively.

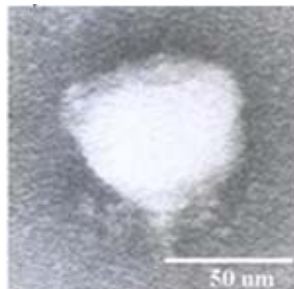


Figure 2.11 – Morphology of φIBB-SL58B for *S. lentus*.

So far, all polyvalent phages reported in literature belong to the *Myoviridae* family of which the most knowns are phage K and phage φ812 that infect several pathogenic staphylococci isolated from both human and bovine infections (Rees and Fry, 1981 and

1983; Pantucek *et al.*, 1998). Thus, phage ϕ IBB-SL58B is the first polyvalent Staphylococcal phage belonging to the *Podoviridae* family.

One-step growth curve

One-step growth curves were performed to analyse the life cycle of phage ϕ IBB-SL58B. The number of free and total phages present in different time intervals was used to estimate the different infection periods (Figure 2.12).

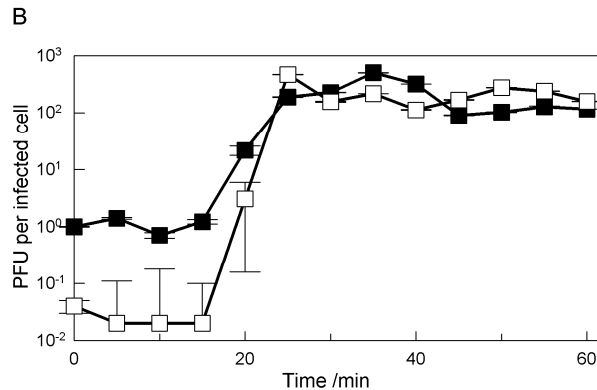


Figure 2.12 – One step growth curve of phage ϕ IBB-SL58B for *S. lentus*.

Phage ϕ IBB-SL58B has a latent and eclipse periods of about 15 min which are followed by a rise period of 20 min that results in a burst size of 507 PFU per infected cell. The life cycle values obtained for ϕ IBB-SL58B differ from the typical values observed for: T7 and N4-like phages. The burst size is higher than in T7, however lower than the burst sizes observed in N4-like phages which are around 3000 after 3 h of post-infection. Also, contrarily to N4-like phages which have a 30 min latent period (Schito, 1974) the phage isolated and characterized in this work has a 15 min latent period. Phage ϕ IBB-SL58B also differs from phi29-like phages which have a burst size of 180 and a 35 min latent period, as well as from P22-like phages that possess burst sizes of 1000 and 30 min latent periods (Yamamoto, 1969). This shows that the phage selected for characterization, ϕ IBB-SL58B, has uncommon values regarding the phage life cycle parameters.

Adsorption experiments

For the estimation of the adsorption rate, the number of free phages was calculated from the PFU of chloroform-treated samples within 15 min after inoculation (Figure 2.13).

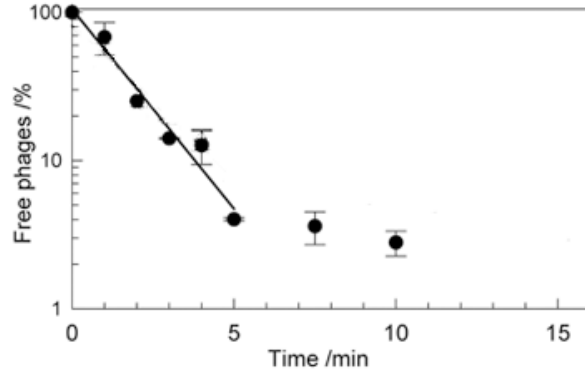


Figure 2.13 – Adsorption of phage ϕ IBB-SL58B to its host *S. lentus* at a MOI of 0.01.

S. lentus phage ϕ IBB-SL58B adsorbs fastly to the host cells and after 5 min the percentage of free phages was already less than 10 % (Figure 2.13). The adsorption rate, calculated according to Hadas *et al.* (1997) was of 1.051×10^7 phages $\text{ml}^{-1} \text{min}^{-1}$.

Analysis of phage structural proteins

Phage structural proteins were analyzed by gel electrophoresis to allow possible comparisons and correlation with other phages. Furthermore, some bands were submitted to N-terminal sequencing for further characterization (Table 2.9).

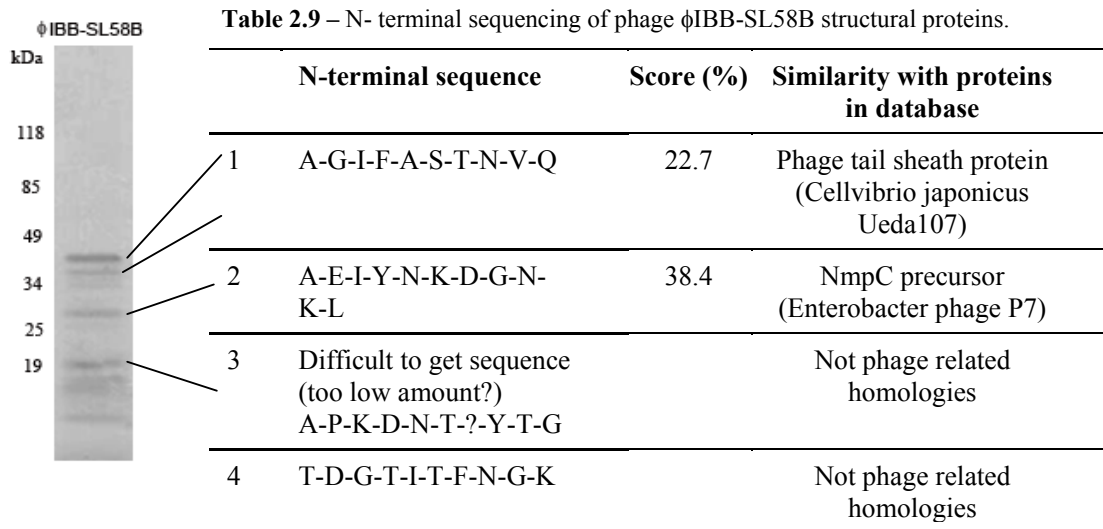


Figure 2.14 – Phage ϕ IBB-SL58B structural proteins.

Phage ϕ IBB-SL58B has 9 visible structural protein bands of which the 47.8 kDa polypeptide appears in greater amount (Figure 2.14). All these bands have sizes smaller than 49 kDa. According to literature, the N4-type phages have 10 structural proteins

where the major component is a 48kDa polypeptide coat protein (10). This similarity between the major polypeptide expressed in ϕ IBB-SL58B 47.8 kDa and the 48 kDa coat protein of N4 phages could be a good reason to classify the *S. lentus* phage within the N4-like phages, however, there is no homology, in terms of N-terminal sequencing of that protein band with the specified phage group. In fact, N-terminal sequencing of four bands did not improve the knowledge on the structural proteins of this phage as basically there was no high homology (scores of 22.7 and 38.4%) between the proteins of this phage and proteins in the database. Also, the proteins identified as 3 and 4 had no similarity to phage proteins. The results of this study also do not help on the characterization of this phage, keeping the classification still a mystery.

Phage DNA studies

The isolated phage ϕ IBB-SL58B DNA was digested with several restriction enzymes. This was performed in order to determine the approximate genome size of this phage. However, the DNA extracted from phage ϕ IBB-SL58B proved to resist cleavage with EcoRI, EcoRV, HindIII and Tsp509I as well as with BamHI, Sau3AI, SmaI, SpeI, SphI and XbaI and therefore the genome size of this phage could not be determined (data not presented). This phage appears to be resistant to cleavage by this wide range of restriction enzymes tested. This fact has been also reported by other authors for N4-like phages (Ohmori *et al.*, 1988; Zehring and Rothman-Denes, 1983).

Genome overview

The studies described above failed to classify phage ϕ IBB-SL58B within a determined *Podoviridae* group. Thus, it can be assumed, based on all previously performed studies that this might be a novel type of phage. Genome sequencing was performed to elucidate on the genes present on this peculiar phage. Intensive sequencing work has been performed and a number of oligos have been designed in order to obtain the left and right terminal repeats of this phage. However, all the experiments performed have not, so far, led to the determination of the complete genome. Nevertheless, the preliminary results obtained are presented in this study (Table 2.10 and Figure 2.15).

Table 2.10 – Gene similarities and properties (Blastp and blastx).

gene	Strand	Left end	Right end	Gene length	Homology	Phage	Score	Expect	Identity %	Accession nr.
1	+	92	271	180	gp0.36	K1F	60.1	5e-08	54	YP_338092
2	+	268	486	219	unknown					
3	+	483	791	308	unknown					
4	+	793	1683	891	unknown					
5	+	1683	2087	405	unknown					
6	+	2089	2295	207	unknown					
7	+	2279	2494	216	gp33	Xop411	36.6	.59	30	YP_001285703
8	+	2481	2783	303	unknown					
9	+	2780	3196	417	unknown					
10	+	3377	3640	264	unknown					
11	+	3640	4116	477	unknown					
12	+	4167	4415	249	DNA primase	LKA1	49.7	7e-05	44	YP_001522861
13	+	4403	4834	432	unknown					
14	+	4842	5804	963	DNA helicase	LKA1	147	1e-37	35	YP_001522864
15	+	5806	6087	282	DNA_B helicase	LUZ19	101	2e-20	50	YP_001671959
16	+	6349	7347	999	DNA ligase	VP4	69.36	4e-10	33	YP_249578
17	+	7436	8032	597	Hyp protein	phiKMV	45.4	0.003	28	NP_877455
18	+	8042	10495	2454	DNA polymerase	LKA1	540	2e-151	40	YP_001522870
19	+	10509	11339	831	Hyp protein	LKA1	91.3	8e-17	32	YP_001522872
20	+	11339	12340	1002	DNA exonuclease	LKA1	132	4e-29	35	YP_001522873

Table 2.10 (Cont.) – Gene similarities and properties (Blastp and blastx).

gene	strand	Left end	Right end	Gene length	Homology	Phage	Score	Expect	Identity %	Accession nr.
21	+	12345	12501	156	Hyp protein	Era103	35.8	0.92	40	YP_001039662
22	+	12498	12896	399	unknown					
23	+	12889	13314	426	DNA exonuclease	LKA1	110	3e-23	46	YP_001522874
24	+	13307	14341	1053	gp23	SP6	284	9e-75	46	NP_853583
25	+	14341	14517	177	unknown					
26	+	14525	14908	384	Hyp protein	BcepF1	73.9	4e-12	50	YP_001039759
27	<150									
28	+	15022	17511	2490	DNA polymerase	MSMB43	427	3e-117	32	ZP_02468154
29	+	17624	17809	186	unknown					
30	+	17802	18224	423	unknown					
31	+	18208	18597	390	unknown					
32	+	18607	20112	1506	Head-to-tail joining	MSMB43	390	2e-106	43	ZP_02468149
33	+	20112	20906	795	Hyp protein	MSMB43	80.5	1e-13	34	ZP_02468148
34	+	20980	22002	1023	Minor capsid protein 10B	MSMB43	245	5e-63	42	ZP_02468147
35	+	22098	22709	612	Tail tub protein A	MSMB43	120	8e-26	36	ZP_02468146

Table 2.10 (Cont.) – Gene similarities and properties (Blastp and blastx).

gene	strand	Left end	Right end	Gene length	Homology	Phage	Score	Expect	Identity %	Accession nr.
36	+	22711	25302	2592	Tail tub protein B	MSMB43	488	1e-135	35	ZP_02468145
37	+	25304	26086	783	unknown					
38	+	26096	28339	2244	Hyp protein	MSMB43	96.3	1e-17	20	ZP_02468143
39	+	28353	32162	3810	Hyp protein	MSMB43	216	2e-53	24	ZP_02468142
40	+	32294	34495	2202	Hyp protein	MSMB43	95.9	1e-17	37	ZP_02468141
41	+	34497	35219	723	unknown					
42	+	35233	35409	177	gp17.5	BA14	33.5	4.7	33	YP_002003494
43	+	35402	35743	342	unknown					

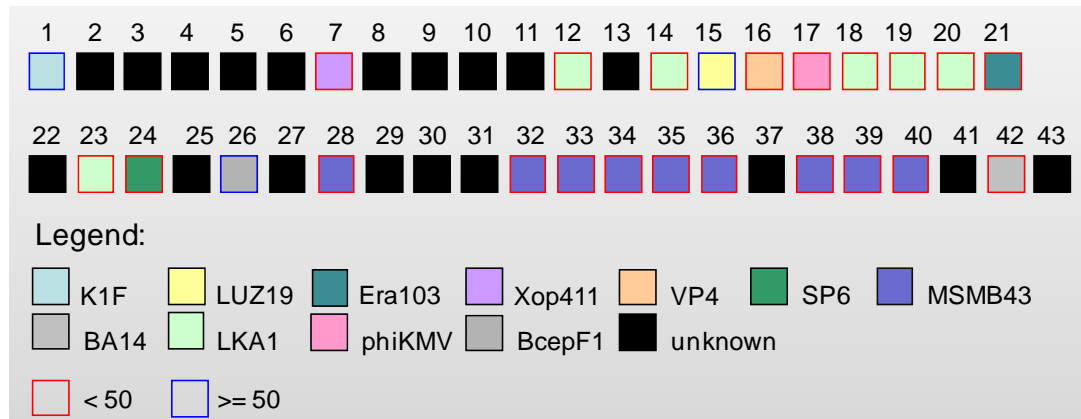


Figure 2.15 – Schematic block representation of the 43 potential genes present in the genome of *S. lentus* phage ϕ IBB-SL58B. Coloured blocks correspond to homology with genes of phages in the database. The red and blue block outlines represents the homology percentage, below and above or equal to 50%, with a gene in the database.

The genome of ϕ IBB-SL58B consists of linear double-stranded DNA and the biggest contig contains 38,762 nucleotides and 43 orfs as predicted using GeneMark software. The GC content of this phage is 56% and no tRNA genes were identified. Also, all genes are transcribed from the same strand. Translated orfs were compared to known proteins sequences within the non-redundant database using Blast tools. The orfs encoding with known proteins are listed in Table 2.10 along with their putative function and the organisms with most similar sequence. Most genes (19 orfs) of ϕ IBB-SL58B present no significant similarities with phage genes in the databank (black blocks in Figure 2.15). Only 3 orfs, the orfs1, 21 and 26, gave homology percentages above or equal to 50%. These 3 orfs showed similarities with genes of three distinct phages: coliphage K1F, *Erwinia emilovara* phage Era103 and the *Burkholderia ambifaria* phage BcepF1. Phages K1F and Era103 belong to the T7 group of *Podoviridae* phages while BcepF1 is a Bcep781-like *Myoviridae* phage. Many orfs of ϕ IBB-SL58B showed low homology percentages (<50%) with 7 different phages of which only one, the *Xanthomonas oryzae* pv. *oryzae* phage Xop411, does not belong to the family *Podoviridae* but instead to the *Siphoviridae* family. So, this study reveals that the *S. lentus* phage ϕ IBB-SL58B has similarities with several genes of gram-negative phages and this fact that has never been reported.

As the information gathered with analysis of the phage genome does not present that much similarities with any phage there was not hypothesized analogies with any phage

arrangements and clustering. Nevertheless, genes from gp0.36 to gp17.5 presented some similarity with early, middle and late genes characteristically observed in T7 phages. Moreover, there was not obtained homology with any RNA polymerase (RNAP). RNAP binds and initiates transcription at specific conserved promoter sequences and possibly the non-identification of this gene was also responsible for the uncharacteristically low number of phage specific promoters identified using PHIRE with a string length of 20, degeneracy of 4 and a window size of 20 (Table 2.11).

Table 2.11 – Promoter sequences and location found in ϕ IBB-PF7A genome.

Nr.	Start	End	Promoter sequence	Mismatch
1	1064	1083	CGTACGTGCAGCGTGACGGC	3
2	9381	9400	AGTACGTGCAGCTGGACAGC	2
3	12607	12626	AGTACGTGCAGCAGGACGGC	0
4	24872	24891	AGTACCGGCAGCGGGACGAC	4
5	26505	26524	AGTACGTGCAGCAGAACCAA	4
Consensus			AGTACGTGCAGCAGGACGGC	

All the promoters identified with PHIRE are not in intergenic regions but rather embedded in orfs, more specifically in orfs 4, 18, 23, 36 and 38, respectively. Therefore, none of these promoters can be considered suitable candidates.

There was identified only one terminator starting at 21,969 nt and with a length of 31 nt, suggesting a weak termination activity. This terminator lies in the minor capsid protein 10B (gp10B) of this phage.

Understanding and classifying phage ϕ IBB-SL58B is proving to be a big challenge as there is no significant morphological, physico-chemical or even genomic similarities with any known phage or group of phages. The genome sequencing is still being performed to further reach the ends of this phage. Nevertheless, the study of the phage genome proves that the assembly has been obtained in the correct order as the first known gene obtained is gp0.36 and the last one from this contig fragment is gp17.5 which is the lysis protein in many phages.

Materials and Methods

Bacteria and growth conditions

The bacterial strains used in this work (see Table 2.7) were previously isolated from a dairy plant (Paços de Ferreira, Portugal). 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, selection and concentration

Bacteriophages were isolated, selected, propagated and concentrated as previously described in Materials and Methods of sub-chapter 2.1.

Phage titration

Bacteriophage titer was analysed as described by Adams (1959).

Bacteriophage adsorption

Bacteria in the logarithmic 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, incubated at room temperature with shaking (150 rpm, Rotamax 120, Heidolph) and samples were collected every minute for 15 min. Samples were immediately chloroform-treated, diluted and plated on TSA plates. After overnight incubation at 30 °C phage plaques were counted.

One-step growth curve

One-step growth curve was performed as described by Pajunen *et al.* (2000) with some modifications as described in sub-chapter 2.1.

SDS-PAGE

Purified phage solution was precipitated with 4 vol of ice-cold acetone and the pellet was air-dried and later 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 (1970). Briefly, 24 µl of sample were added to 8 µl of 4 × Laemmli buffer and

boiled for 10 min. Samples were then loaded to a 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

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

Restriction enzyme digestion

Phage ϕ IBB-SL58B DNA was digested with the following restriction enzymes: Eco RI, Eco RV, Hind III, Tsp, Bam HI, Sau 3AI, Sma I, Spe I, Sph I and Xba I, 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.

Electron microscopy

Bacteriophage particles were sedimented at 25,000 \times 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 (pH 7.2), and examined in a Philips EM 300 electron microscope (performed by Dr. H. W. Ackermann, Laval University, Quebec, Canada).

Phage DNA sequencing

Phage ϕ IBB-SL58B DNA was sheared with a nebulizer and sizes between 1 and 4 kb were cut from a 1% 1 \times TAE agarose gels after overnight electrophoresis at 30V. Afterwards, the fractions were purified, end repaired and ligated overnight at 16°C into pZero cloning vector (Zero background/Kan cloning kit, Invitrogen) which had been previously digested with EcoRV. After overnight ligation, chemical transformation to One Shot® TOP10 cells (Invitrogen) was performed. The cells were recovered in SOC medium, incubated for 1 h at 37°C and 225 rpm (Titramax plate shaker, Heidolph) and

afterwards plated onto a LB-Kan containing X-Gal plate. Selected clones were picked with a colony picker (QPix2) and grown overnight in LB medium at 37 °C and 225 rpm. The cultures were centrifuged and plasmid DNA was extracted and purified and eluted in $0.1 \times$ TE buffer. PCR was performed with T7, SP6 primers or with oligos designed throughout the sequencing experiments. For sequencing, Big Dye chemistry was used and sequencing was carried out in ABI 3700 DNA Sequencer (Applied Biosystems). Sequence reads were assembled using GAP4 (Staden package software). Sequences were assembled into contigs, and gaps were linked through a primer-walking technique with 20-mer oligonucleotide primers and pure ϕ IBB-SL58B DNA until all contigs assembled into a single sequence.

ORF prediction and annotation

Prediction of open reading frames (orfs) was performed using GeneMark.hmm, OrfFinder and by visual inspection of the sequence searching ATG as start codons. For tRNA gene identification, the tRNAscan-SE program was used. Translated orfs were compared with known protein sequences using Blastx and Blastn, using the non-redundant public GenBank database.

Molecular masses and isoelectric points of phage ϕ IBB-PF7A translated putative proteins were determined with ExPasy. Promoter predictions were calculated using promoter predictor (http://www.fruitfly.org/cgi-bin/seq_tools/promoter.pl).

A search for transmembrane helices was done by using the TMHMM program.

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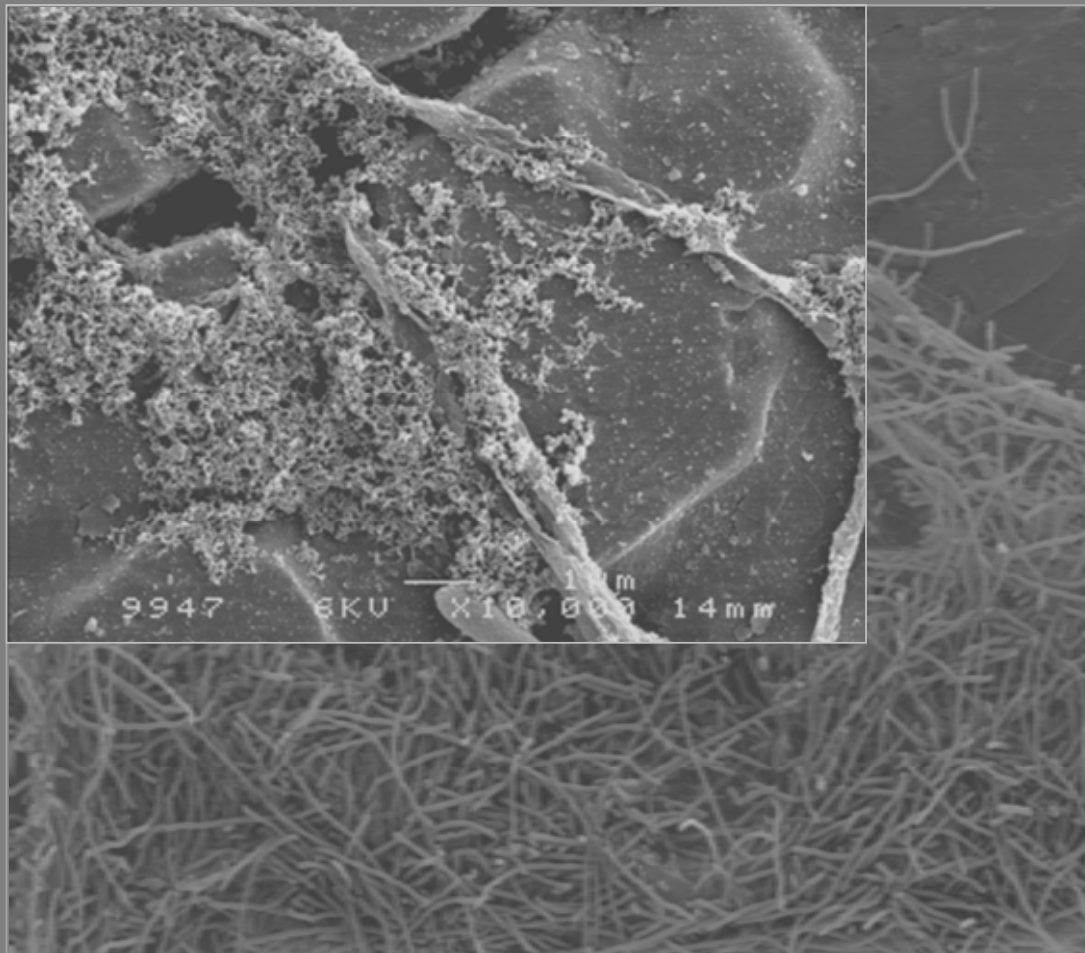
1. LITERATURE REVIEW

2. ISOLATION AND CHARACTERIZATION OF PHAGES

3. PHAGE BIOFILM INTERACTION

4. PHAGE AND PLANKTONIC CELLS

5. MAIN CONCLUSIONS AND SUGGESTIONS FOR FORTHCOMING WORK



3.1 *Pseudomonas fluorescens* biofilms subjected to phage ϕ IBB-PF7A

Summary

Background: *Pseudomonas fluorescens* is an important food spoilage organism, usually found in the form of biofilms. Biofilms are inherently tolerant to antimicrobial agents, therefore alternative methods to biofilm eradication, such as bacteriophages (phages) have been suggested. Phage behavior on biofilms is still poorly investigated and needs further understanding. This work describes the application of phage ϕ IBB-PF7A, a newly isolated phage, to control *P. fluorescens* biofilms. The biofilms were formed under static or dynamic conditions and with or without renewal of medium.

Results: Conditions for biofilm formation influenced the feature of the biofilm and the morphology of *P. fluorescens*. Biomass removal due to phage activity varied between 63 and 91 % depending on the biofilm age and the conditions under which the biofilm had been formed and phages applied. Removal of the biofilm by phage treatment was faster in younger biofilms, but interestingly, after only 4 h of treatment approximately the same number of surviving cells was detected in all tested biofilms, even in older biofilms. Under static conditions, a 3 log higher number of phage progeny remained either inside the biofilm matrix or attached to the substratum surface than under dynamic conditions, pointing to the importance of experimental conditions for the efficacy of phage entrapment into the biofilm.

Conclusions: Phage ϕ IBB-PF7A is highly efficient in removing *P. fluorescens* biofilms within a short time interval. The conditions of biofilm formation and applied during phage infection are critical for the efficacy of the sanitation process. The integration of phages into the biofilm matrix and their entrapment to the surface may be further beneficial factors when phage treatment is considered alone or in addition to chemical biocides in industrial environments where *P. fluorescens* causes serious spoilage.

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Background

Pseudomonas fluorescens is a spoilage causing bacterium present in a variety of food related environments. In dairy industry, *P. fluorescens* is one of the most commonly isolated psychrotrophic bacteria that dominate the microflora of raw or pasteurized milk at the time of spoilage [1-6]. This spoilage ability is mostly due to the capability of producing heat-stable extracellular lipases, proteases and lecithinases that survive the thermal processing steps [2, 6-8]. This bacterium is also one of the three most predominantly isolated bacteria associated with spoilage of fresh poultry and reports of spoilage due to *P. fluorescens* date since the 1930s [9-12]. More recently, some studies have revealed that some strains of *Pseudomonas* can increase the colonization of inert surfaces by *Listeria monocytogenes* [13] and/or protect this pathogenic bacterium from disinfectants [14]. There are also studies reporting spoilage of refrigerated foods with *P. fluorescens*, in particular of refrigerated meat products [15-17]. The contamination of the meats results in changes in appearance and odour during prolonged storage. Furthermore, *P. fluorescens* is also recognized to be a model organism for biofilm studies as it can easily form biofilms in different laboratory simulators [18-20].

The interest in applied bacteriophage research has increased during recent years mainly due to positive results obtained with phage therapy applied to animals [21-24]. Also phage application to certain meat products was allowed, since August 2006, by the United States Food and Drug Administration (FDA) in order to control *Listeria monocytogenes* [25]. There is also an increase in the number of patents of application of phages to control pathogenic bacteria in industrial environments and foodstuffs [26-29]. The idea in this area is to either keep the biofilm propagation limited by the phages over long times, e.g. by insertion of phages in surface layers (e.g. [30]) or to apply phages at different stages of production and processing to reduce food product contamination at that point or to protect against contaminations at subsequent points, which can be performed also in combination of sterilizing chemical agents as long as these agents do not reduce the biological activity of the phages [29].

Biofilm phenomena have been studied over many years and it is generally recognized that this bacterial lifestyle on surfaces is dominant. Although many studies involve phage infection of cells, most of them only consider planktonic bacteria. Bacteria attached to surfaces have totally different characteristics such as they are embedded in a matrix

composed of exopolysaccharides, proteins and nucleic acids and the cells represent different growth stages. As phage infection and life cycle generally strongly depend from the growth stage of the host bacterium (see e.g. [31, 32]) the treatment of slowly growing cells in biofilms is a challenge. Some studies have already been made regarding the application of phages to eradicate bacteria in the form of biofilm [30,33-38] nonetheless more understanding of phage action in biofilm influenced by age and formation conditions is still required.

In a previous work with biofilms [37] it was already showed that under optimal conditions phage ϕ S1 could infect well *P. fluorescens* biofilms. This was a study performed with a culture collection host and phage system that had been isolated from soil. In this work, a *P. fluorescens* isolated from a dairy industry was used. The bacterium was isolated from a biofilm present on the stainless steel teatcup shell which indicates that this isolate would easily form biofilms on stainless steel slides. The aim of this work was to study the application of the recently isolated T7-like bacteriophage ϕ IBB-PF7A [39] to control *P. fluorescens* biofilms. Aside from a very high efficiency of this phage in biofilm removal, different conditions applied for biofilm formation and during phage application proved their influence on the time kinetics of the phage absorption and biofilm removal process. Interestingly however, the single species model biofilm was efficiently removed under all conditions which suggests phage ϕ IBB-PF7A to be a superior sanitation agent.

Results

Biofilms formed under different conditions

In this work, *P. fluorescens* biofilms were allowed to form onto stainless steel (SS) slides, which is a common substratum used in the dairy industry. Three different experimental approaches for biofilm formation were applied: (i) static conditions, i.e. no shaking applied, with media renewal every 12 h (SR), (ii) dynamic conditions, i.e. incubation on an orbital shaker, with media renewed every 12 h (DR), and (iii) dynamic conditions without renewal of cultivation medium (DNR) (see Methods for description of the different conditions). Also, different biofilm ages were studied ranging from 24 up to 168 hours.

The number of cells present on SS slides was assessed after different periods of biofilm formation (Figure 3.1). Lowest cell counts, detected as colony forming units after removal of the cells from the substratum, were obtained from biofilms which were incubated under shaking conditions (dynamic conditions) with an additional renewal of the cultivations medium. Dependent on the incubation period, 10 to 100 times higher cell numbers were detected from coupons which were incubated without any turbulence, i.e. under static (SR) conditions (Figure 3.1). Interestingly, the difference in the number of colony forming units between the different conditions decreased with the time of incubation.

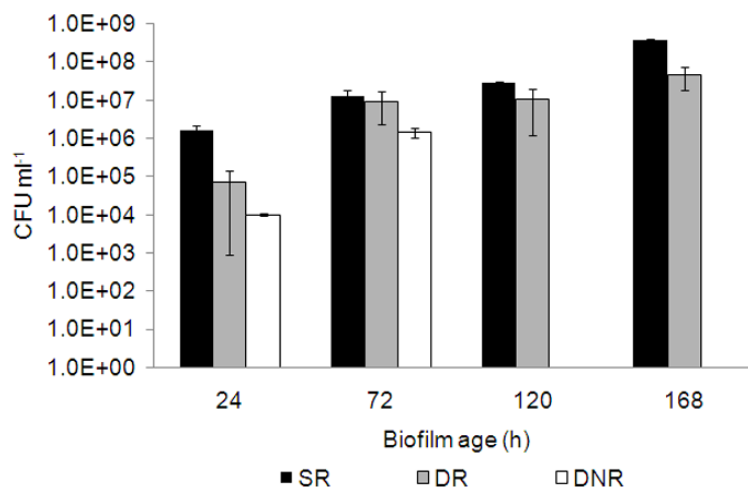


Figure 3.1 - Number of biofilm cells present on stainless steel slides. *P. fluorescens* biofilms were formed during different times on stainless steel slides under different conditions: static with media renewal (SR), dynamic with media renewal (DR), dynamic non-renewal of media (DNR). For all experiments $n = 6$. Error bars indicate standard deviations.

Additionally to the determination of colony forming units, also the dry weight of the biofilms was analyzed. These dry weight measurements cannot be directly correlated with viable cell numbers (Table 3.1). For example, the viable cell counts obtained from biofilms which were cultivated for 72 h under static or dynamic conditions (DR and SR) were practically identical, but the determined dry weight was clearly different. This difference in dry weight is most likely due to different amounts of EPS matrix present in these different biofilms.

In order to better characterize the effect of dynamic conditions on the biofilm, samples after incubation for 24 to 168 hours were analyzed by field emission scanning microscopy (FESEM) (Figure 3.2). The FESEM micrographs show a predominance of filamentous cells in early stage biofilms (24 h and 72 h) and a shift to rod-shape forms in older biofilms (120 h and 168 h).

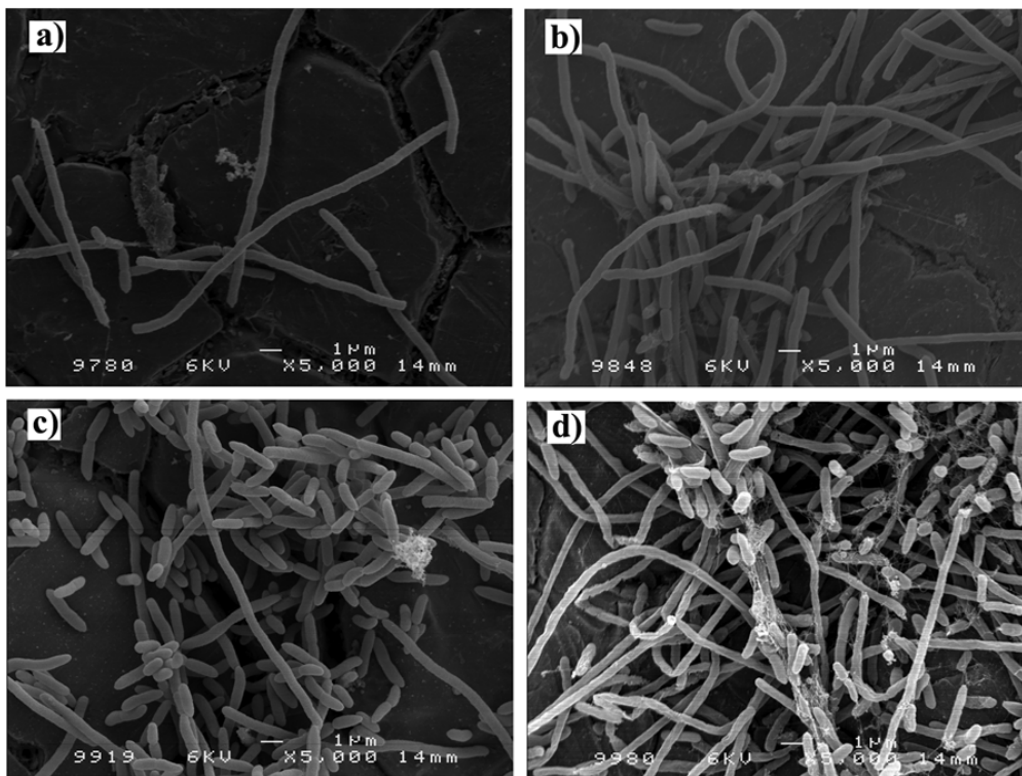


Figure 3.2 - Dynamic *P. fluorescens* biofilms formed on stainless steel slides with media renewal every 12 h. FESEM micrographs before phage application: a) 24 h; b) 72 h; c) 120 and d) 168 h old biofilm.

For the dynamic conditions without renewal of medium biofilm formation was studied only during 24 h and 72 h, as there was clearly decreased biofilm formation ability, most likely due to the lack of nutrients.

Overall, this study shows that the biofilm formation conditions influence the number of cells and biomass present on the coupons as well as the *P. fluorescens* cell morphology. This study is an important basis for the valuation of the effects of bacteriophages.

Efficacy of phage ϕ IBB-PF7A to control biofilms

Single species biofilms do not mimic real conditions, but these biofilms determinedly present advances in the biofilm-phage interaction research field. Here it is investigated how effective the recently isolated T7-like bacteriophage ϕ IBB-PF7A is in controlling biofilms under static and dynamic conditions. In these experiments the number of phages applied was 1×10^7 PFU ml⁻¹ as this concentration is in the range of normally used phage solutions (1×10^6 PFU ml⁻¹ to $\times 10^{10}$ PFU ml⁻¹) [33,36-38].

Initial tests performed with biofilms and phage ϕ IBB-PF7A showed that maximum cell reduction was achieved within 4 hours. Therefore, the effect of the phages was followed over a time interval of two and four hours respectively, which is clearly lower than the 24 hours infection period used in other biofilm-phage studies [38, 40-42].

As a negative control, biofilms immersed for four hours in a solution of SM buffer and TSB without addition of phages also investigated in order to verify that the observed effects are really related to the phages and not to a detachment of the biofilms as a response to the addition of new SM medium.

In order to evaluate the phage ability to infect the different *P. fluorescens* biofilms described above, samples were taken after two and four hours for cell and phage enumeration. Already two hours after phage addition, a very strong reduction of the number of cells on the SS slides was observed for all the different biofilms (Figure 3.3). In older biofilms (120 h and 168 h) the phage acted somewhat slower, obviously older, stationary phase biofilms delay the cell lysis process. However, under all tested conditions, a 3 to 5 log order reduction of the cell count was detected during only four hours of phage exposure.

Biofilm cell reduction was about one order of magnitude higher in static conditions compared to dynamic conditions, despite of the clearly different cell morphology (compare Figures. 3.3a and 3.3b). Furthermore, it was also wanted to investigate whether the application of the phages would lead to the release of cells.

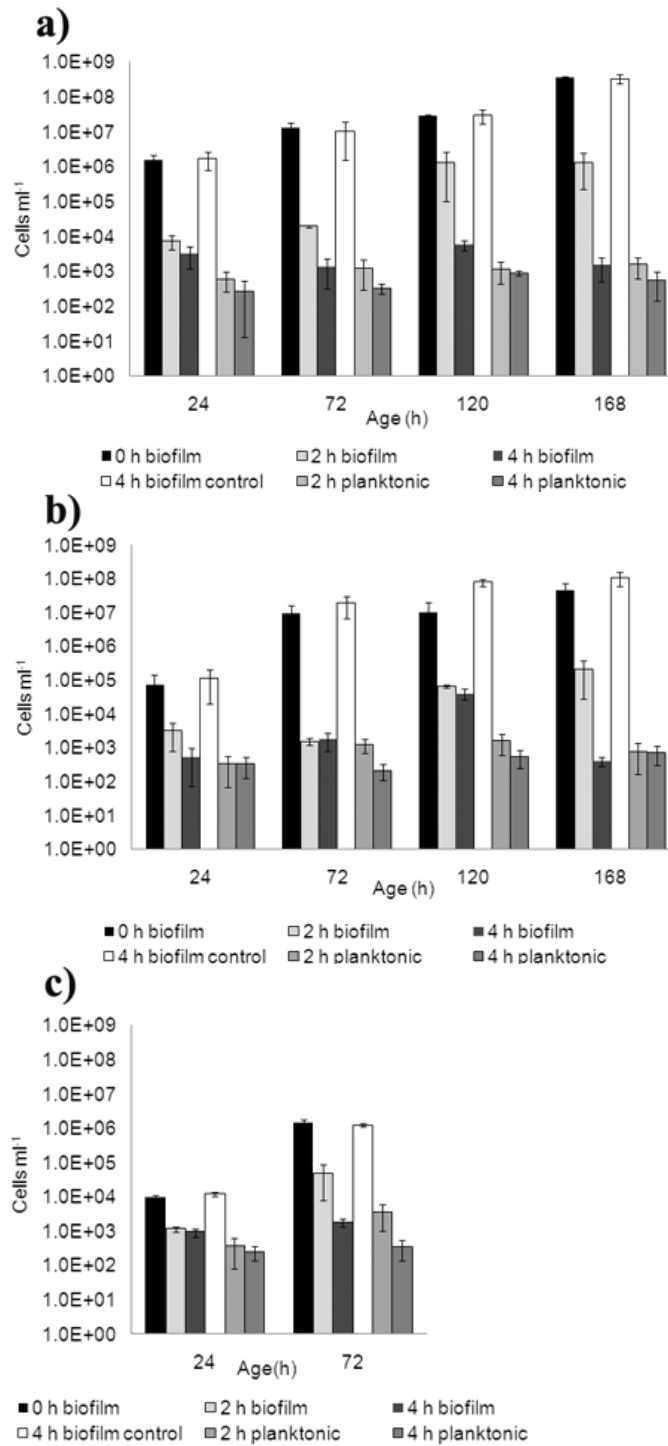


Figure 3.3 - Number of *P. fluorescens* biofilm and planktonic cells before and after exposure to phage ϕ IBB-PF7A. Cells before phage (time 0 h), control experiments performed for 4 h in SM buffer-TSB, phage exposure for 2 h and 4 h are presented in biofilms were formed under: a) static with media renewal (SR), b) dynamic with media renewal (DR), and c) dynamic with non-renewal of media (DNR). For all experiments n = 6. Error bars indicate standard deviations.

Measurements of cell numbers in the planktonic phase showed that in phage infection experiments there was a detachment of cells or clusters of cells into the liquid medium, although these numbers were quite low, generally below 10^3 cells per ml. The measurements of the control coupons show that the biofilms are not detached due to the half-strength TSB and the cell numbers are similar to those at the start of the experiments (Figure 3.3) and therefore it can be concluded that the detachment in phage infection experiments is only due to the presence of phages.

The number of phages in the planktonic phase was approximately the same after infection of the different biofilms (Figure 3.4). The main difference in phage counts was related to phages adsorbed to the biofilms and SS slides. A higher number of phages adsorbed to the biofilms and SS slides under static conditions. However, infection under dynamic conditions decreased the ability of phages to adsorb to the substratum and remaining biofilms. Despite this, it was surprising that the number of phages found to be absorbed is approximately in the order of magnitude of the phages applied for the treatment, which indicates a very efficient reproduction of the phages.

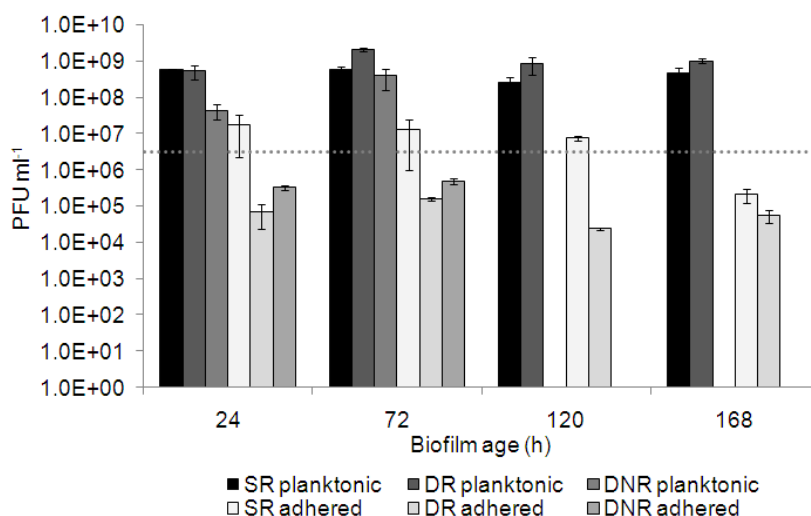


Figure 3.4 - Number of phages used to infect the different biofilms and progeny phages adsorbed and released after 4 h of infection of biofilms. Horizontal line: initial number of phages used. Static biofilms with media renewal (SR), dynamic biofilms with media renewal (DR); dynamic biofilms without media renewal (DNR). For all experiments $n = 6$. Error bars indicate standard deviations.

FESEM micrograph taken after 30 min of exposure of a 24 h static biofilm to phage ϕ IBB-PF7A shows some individual rod-shaped cells as well as a vast number of phages adsorbed to the SS slides and to the bacteria. The phages appear either individually or as groups of phages (Figure 3.5).



Figure 3.5 - *P. fluorescens* cells and ϕ IBB-PF7A phages on stainless steel slides. FESEM micrograph taken after infection 30 min of infection of a 24 h static *P. fluorescens* biofilm formed with media renewal every 12 h.

Table 3.1 - Biomass before and after 4 h of phage application to different biofilms and biomass removal percentages.

	Biofilm age (h)	Biomass start (0 h) (\pmSD)	Biomass end (4 h phage) (\pmSD)	Biomass removal (%)
SR	24	8.50 (0.71)	1.50 (1.08)	82.35
	72	12.50 (3.54)	3.00 (1.73)	76.00
	120	13.67 (4.73)	5.00 (1.15)	63.42
	168	14.67 (3.79)	4.00 (3.06)	72.73
DR	24	5.00 (1.41)	1.00 (0.00)	80.00
	72	7.50 (0.71)	0.67 (0.58)	91.07
	120	8.67 (0.50)	2.00 (1.46)	76.93
	168	12.50 (3.21)	4.33 (1.15)	65.36
DNR	24	7.33 (2.08)	2.00 (1.52)	72.71
	72	11.00 (1.73)	3.00 (2.06)	72.72

SD means standard deviations from 6 different SS slides.

Table 3.1 shows the dry weight on the slides before and after four hours of phage application and the relative biomass decrease after infection of different biofilms. Four hours after infection a considerable biomass decrease was observed for all biofilms. The relative biomass removal accounted for 63 to 91 %. In general, older biofilms (120 h and 168 h) showed a lower biomass decrease, most probably due to a higher amount of

cellular debris that stayed attached to the SS surfaces (Figure 3.6). This cellular debris and dead cells still constitute important sites for phage attachment.

Four hours after phage application, in all experiments, cells from the remaining biofilms were isolated and tested for resistance to the stock phage solution. In none of these tests was found phage-resistant bacteria, indicating that the time of the experiment was not long enough for the bacteria to acquire any form of resistance (data not shown) as described previously by other authors [38, 43].

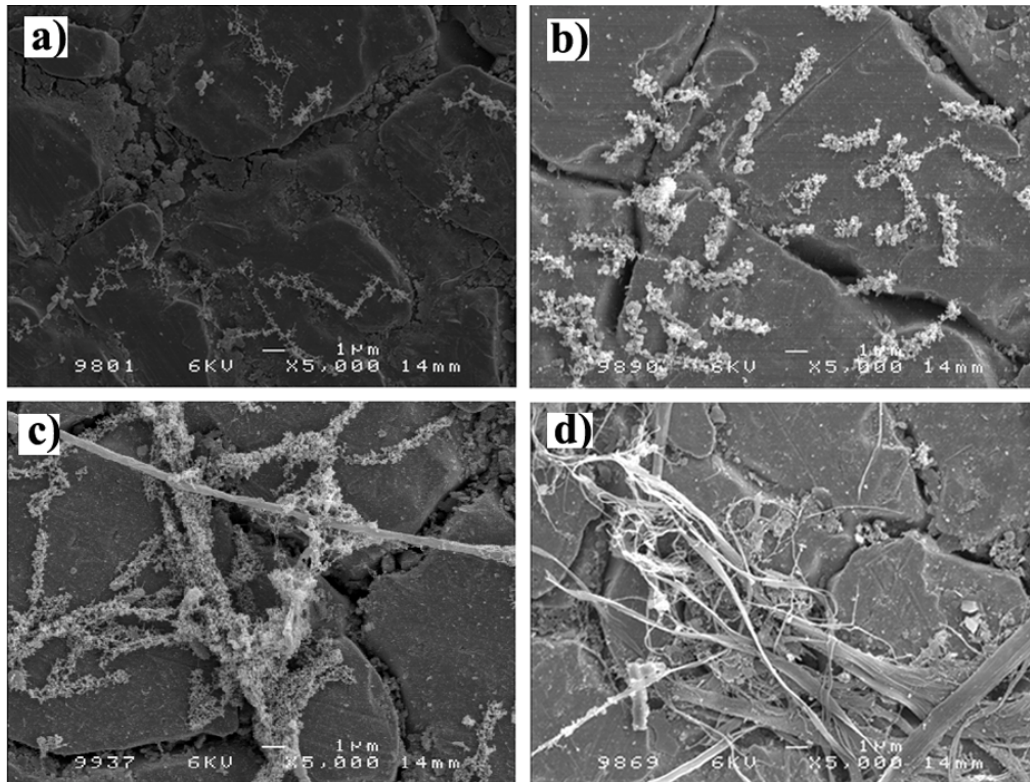


Figure 3.6 - Cellular debris and biofilm remains on stainless steel slides after 4 hours of phage ϕ IBB-PF7A infection of dynamic *P. fluorescens* biofilms formed with renewal of media every 12 h. FESEM micrographs: a) 24 h; b) 72 h; c) 120 and d) 168 h old biofilm.

Phage ϕ IBB-PF7A is a potential efficient biological agent to control biofilms as the phage's action on biofilms resulted in good biofilm cell log decreases factors and high percentual biomass removal.

Discussion

P. fluorescens is a known milk product contaminant being able to produce extracellular enzymes that spoil milk products. In this work *P. fluorescens* biofilms were challenged with a newly isolated lytic phage. The data presented here, shows the potential of the novel phage ϕ IBB-PF7A for controlling and reducing the *P. fluorescens* biofilms formed under three different conditions. Phage ϕ IBB-PF7A was able to reduce greatly the number of biofilm cells present on stainless steel slides already after 2 h of infection experiment and this reduction was even more noticeable after 4 h of phage application. The biofilm cell log reduction factors, after 4 h of phage exposure varied between 3 and 5. Complete eradication was not achieved in any of the studied cases, however it is evident that phage ϕ IBB-PF7A can greatly decrease the number of biofilm cells present on the surfaces. Similar cell log reductions have been reported in other biofilm-phage experiments, however in all these other studies the phage infection period used was 24 h [38, 40-42]. This shows that phage ϕ IBB-PF7A has very good potential as a biofilm control agent as it can achieve the same result in only 4 hours.

In this study, the formation of biofilms on substrata immersed on microplates does not imitate true conditions observed in a variety of environments; nevertheless it is a simple, rapid and reproducible method with which it is easy to assess the influence of different parameters. Biofilms formed show that *P. fluorescens* are able to grow either as rod-shaped or as filamentous like form. This change in morphology is due to the rotation applied (100 rpm) to form dynamic biofilms. However, there is a clear switch on the cell morphology: young biofilms (24 h and 72 h) are predominantly filamentous like, while older biofilms (120 h and 168 h) consist of more rod-shaped cells (Figure 3.2). Unlike in *P. aeruginosa* and *P. putida* where this filamentous cell morphology has already been described [44-46] the filamentous morphology has not been reported for *P. fluorescens*.

Biofilm cell lysis starts faster under dynamic than under static conditions, but interestingly, the total relative biofilm reduction after four hours was not as effective in dynamic biofilms compared to static biofilms. Under dynamic conditions, phages probably meet their hosts faster than on static conditions, indicating the important role of the convection mechanism. Conversely, under static conditions, the lack of agitation keeps the progeny phages in the proximity to other neighboring biofilm cells. Under static conditions, the new phages, which are released due to phage infection and lysis of the

host cells, are not transferred to the bulk and this enhances the biofilm cell lysis. This hypothesis is confirmed by the values of adsorbed phage counts which were more than two orders of magnitude higher under static conditions compared to dynamic conditions ($\sim 1 \times 10^7$ and $< 1 \times 10^5$ PFU ml⁻¹ respectively). In both situations the phage numbers in the planktonic phase were approximately the same (1×10^9 PFU ml⁻¹). Nevertheless, it is important to state that the same efficiency of cell lysis was reached within four hours after phage infection under dynamic and static conditions.

Biofilm formation is directly related to the supply of nutrients [47-51]. In this work, *P. fluorescens* biofilm formation under non-renewed media conditions was the worst and least effective method for producing biofilms (Figure 4.1). This lower ability of forming biofilms might be due to depletion in nutrients available for the sessile bacterial cells, which were consumed by the planktonic cells rendering difficult the multiplication of the biofilm bacteria. Cerca *et al.* [52] observed also this enhanced biofilm formation under fed-batch (nutrient renewal) conditions in comparison to batch (without nutrient renewal) formation of *S. epidermidis* biofilms. The efficiency to remove biofilms by phages is decreased in biofilms where a change of the medium was not performed. There may be various reasons, including a decreased number of daughter phages and longer phage development cycles in starving bacteria [31, 53], but also less efficient phage adsorption.

Biofilms are composed of pores and channels [54] through which nutrients reach cells present on different layers. It may be also assumed that phages are able to circulate through these channels and pores and by this way reach and adsorb to cells on different biofilm layers, including the basal layer of the biofilm. This study shows that phage application to biofilms resulted in a release of cells to the planktonic phase. This is most likely due to the infection of cells at different layers which causes the sloughing off of parts of biofilms to the liquid medium. After the release of these biofilm clusters to the planktonic phase, the detached biofilm cells are most likely attacked by phages present on the liquid medium as in all situations the number of cells detectable on the planktonic phase was always below 10^3 cells ml⁻¹.

It has been reported that phage aggregation and fixation on surfaces is a mechanism of protection that phages adopt as a strategy of survival from inactivating environmental stresses [54-57]. In this work it is shown that phage ϕ IBB-PF7A in fact adsorbs to stainless steel and phages are found individually or in aggregates (Figure 4.5). Phage

adsorption was clearly dependent on the infection conditions applied. Static conditions allow the deposition of phages while dynamic conditions suppress phage fixation.

Coexistence of phages and bacteria has frequently been reported and discussed in relation to bacterial resistance of the bacteria to the phages as well as a consequent mutation of phages [58]. In this work, phage action on the biofilms formed was studied merely for 4 h which was not a period long enough for the emergence of resistance. Possibly longer periods of phage action on the biofilms would result in the appearance of phage resistant bacteria.

Tail *et al.* [38] has obtained complete eradication of 24 h biofilms of *E. cloacae* when exposing the biofilms during 24 h to a solution of 3 different phages. Curtin and Donlan [30] have adopted a different approach of phage application in order to combat *S. epidermidis* biofilm formation onto catheters. Instead of treating biofilms once they were already present, these authors have adopted pre-treatment of catheter surfaces with phages to decrease bacteria adhesion to the surface. In food industry, pre-treatment of all surfaces is more complicated, so the combination of phages in a cocktail to achieve biofilm eradication is more realistic. Furthermore, there might be attractive scenarios where phage sanitation is applied before or with chemical biocides, which may make sanitation more efficient and therefore decreasing the necessary amount of chemicals.

Phages have been suggested as sanitation agents for different bacteria present in industrial facilities [26-29, 54]. The phage used in this study, ϕ IBB-PF7A, is an interesting biological agent as it has a great ability of lysing biofilm cells in an exceptionally rapid time.

Conclusions

Phage ϕ IBB-PF7A may prove to be useful for the biocontrol of *P. fluorescens* strains in dairy and other food related industries. In laboratory studies it was highly efficient in reducing the number of cells present even in mature *P. fluorescens* biofilms, formed under different conditions, which persuaded to continue further biofilm and phage studies including studies with mixed biofilm communities.

Material and Methods

Bacteria and growth conditions

P. fluorescens was isolated from a Portuguese dairy plant located in Paços de Ferreira and 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

Bacteriophage ϕ IBB-PF7A was isolated from a sewage treatment plant in Esposende (ETAR de Esposende, Portugal). This phage was chosen amongst different *P. fluorescens* phages isolated as it was able to infect a higher number of *P. fluorescens* isolated from a dairy industry [39].

Bacteriophage propagation and concentration

Concentrated phage solutions were produced using the plate lysis and elution method as described by Sambrook & Russel [59] with some modifications. Briefly, a top agar was prepared containing 1 ml of phage solution and 1 ml of a bacterial overnight culture in 300 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 of stock solution

Bacteriophage titer was analysed as described by Adams [60]. Briefly, 100 μ l of diluted phage solution, 100 μ l 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.

Biofilm formation

Biofilms were formed on stainless steel (SS) 1 × 1 cm slides for different time periods (24 h up to 168 h) according to the method described by Cerca *et al.* [52] with some alterations. Briefly, SS slides were placed on the wells of a 6 well microplate containing each well 6 ml of TSB medium. Bacterial culture (50 µl) with an OD₆₀₀ of 1.0, which corresponds to approximately 1.79×10^9 cells ml⁻¹, was added to each well and the microplate was incubated at 30 °C under different conditions. Under static conditions, the 6-well microtiter plates were put in an incubator and the biofilms were formed without agitation and with a change of medium every 12 h during the whole duration of the experiment. Under dynamic conditions, the 6-well microtiter plates were put on an orbital shaker at a constant speed of 100 rpm and two different biofilm formation strategies were studied – with the change of medium every 12 h and without the change of medium. Abbreviation will be used for the different biofilm formation conditions: static conditions with media renewal every 12 h (SR), dynamic conditions with media renewal every 12 h (DR) and dynamic conditions without renewal of media (DNR).

Biofilm infection

Biofilms were allowed to form on stainless steel (SS) slides for different times and conditions in 6-well microplates. Afterwards, the SS slides with biofilm were immersed twice in PBS and placed in new microplates with 3 ml of TSB and 3 ml of phage solution with a concentration of 10^7 PFU ml⁻¹. The 6-well microplates were incubated at 30 °C the same conditions at which the biofilms were formed. Control experiments were performed at the same conditions with the SS slides put, after immersion in PBS, in new microplate wells with 3 ml of TSB and 3 ml of SM buffer. Biofilms before and after phage infection were analyzed for number of cells (CFU counts) and phages (PFU counts) attached to the surfaces and on the planktonic phase as well through dry weight determinations (see below).

CFU and PFU counts of biofilm samples

The number of bacteria and phage present on the SS slides before and after infection of biofilms formed under different conditions was enumerated in order to estimate the efficiency and adsorption characteristics of the phage. Therefore SS slides with biofilms were washed twice by immersion in PBS and afterwards put in 50 ml tubes containing 6

ml of 0.9 % saline solution. The tubes were thoroughly mixed (vortexed 4 × 30 sec) and serial dilutions were immediately performed in 0.9 % saline solution for CFU counts and in SM buffer for PFU counts. For CFU counts the samples were immediately plated on TSA plates and for PFU counts samples were immediately plated using the method described above for phage titration. Six independent parallels were performed for the different countings.

CFU and PFU counts of planktonic samples

Samples for planktonic CFU and PFU counts were removed from the 6-well microtiter plates and serial dilutions were performed. All samples were immediately processed. Six independent parallels were performed for the different countings.

Biofilm dry weight determination

Biofilm dry weight determinations were performed as described by An & Friedman [61]. Briefly, stainless steel (SS) slides with biofilms formed under different conditions were removed from the microplates and rinsed by immersion in PBS. Afterwards, the slides were dried at 100 °C for 24 h and weighed. The SS slides were then carefully washed, dried again for 24 h at 100 °C and weighed (empty control). Biofilm dry weight was calculated from the difference between these measurements. Biofilm dry weight determinations represent six independent parallels for controls (time 0 of infection) and for phage treated slides (4 h after infection).

Resistance assays

Biofilm cells that remained on the stainless steel surfaces were analysed for resistance as described by Sillankorva *et al.* [32] with some alterations. Briefly, swabs were used to collect the bacteria and put on tubes containing 1 ml of saline 0.9%. After, dilutions were made and plated on TSA dishes. After overnight incubation, 20 colonies from different dishes were picked and grown on flasks with 50 ml TSB medium for 10 h. Bacterial lawns of these cultures were done and tested for resistance using the spot test with the phage stock solution. The dishes were incubated for 18 h at 30°C and checked for presence of phage plaque.

Field Emission Scanning Electron Microscopy (FESEM)

Samples were taken before phage infection and after 4 h of phage infection. The SS slides were rinsed by immersion in TSB media before adding 2.5 % glutaraldehyde and incubation at 4 °C for 1 h. Dehydration was carried out with an ethanol series from 30 % to 50 % to 70 % to 80 % to 90 % and absolute, followed by critical drying (Critical Point Dryer CPD 030). Biofilms were coated with platinum coating and analyzed with FESEM in a JEOL JSM-6300F (Tokyo, Japan) instrument.

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3.2. Dual species biofilms of *P. fluorescens* and *S. lentus* challenged with lytic phages

Abstract

*Despite the enthusiasm and increase in phage research in the last years, there are limited studies of phage interaction in dual species biofilms. This work characterizes dual species bacterial biofilms formed by Gram-negative (*Pseudomonas fluorescens*) and Gram-positive bacteria (*Staphylococcus lentus*), and their infection with phages. Dual species biofilms were exposed to a phage cocktail for each species present or to a single phage (ϕ IBB-PF7A) for the less predominant bacterium (*P. fluorescens*). Infection with the phage cocktail was very effective and the biofilms were well removed from the substratum. Additionally, the phage cocktail also controlled the bacteria released from the biofilms to the planktonic phase. Regardless of the low amounts of *P. fluorescens* observed in dual species biofilms, this study shows that phage ϕ IBB-PF7A can easily reach its' target host. After 4 hours of ϕ IBB-PF7A application to the biofilm consortium, the number of *P. fluorescens* cells on the biofilms was highly reduced and there was an increase of the planktonic cell quantity from 2 to 4 hours, most likely due to the release of *S. lentus*, the non-susceptible host, from the partially damaged biofilms. This study evidences that phages are well capable of reducing the target hosts present in dual species biofilms, however phage treatment depends of the application strategy employed.*

Introduction

Microorganisms living attached to inert surfaces in multicellular consortia are known as biofilms. In biofilms the microorganisms are found embedded in a matrix where extracellular polymeric substances (EPS) are considered the primary matrix material (Flemming *et al.* 2000). The wet weight of mature biofilms consists of approximately 90-97 % of water, 2-5 % of microbial cells and 1-5 % of EPS (Zhang *et al.* 1998). The structure of a biofilm depends of several factors such as: the microorganisms present, their physiological state, the physical environment (availability of nutrients and oxygen, flow velocity of the surrounding liquid), and the surface where the biofilm is formed (Sutherland 2001).

Studies of interspecies biofilms have shown that the interactions of species within biofilms influence the susceptibility of the biofilms to antimicrobial agents (Bourion & Cerf 1996; Budhani & Struthers 1998; Cowan *et al* 2000; Erb *et al* 1997; Leriche *et al* 2003; Skillman *et al* 1999). The efficacy of chemical agents in these biofilms is clearly decreased which leads to reduced microbial eradication efficiencies. Microbial protection against chemical agents is a serious problem and it is suggested to be due to enzyme complementation (Shu *et al* 2003) and organized spatial distribution of the cells in the biofilms (Cowan *et al* 2000; Leriche *et al* 2003). Therefore, besides the antimicrobial agent protection advantage, when microorganisms live in biofilm communities, there are other beneficial interactions to one or more strains or species in interspecies biofilms which include conjugation (Ghigo 2001) and coaggregation of cells (Rickard *et al* 2003; Sharma *et al* 2005; Tait *et al* 2002). Antagonistic interactions in multi-species biofilms can include the production of bacteriotoxins (Rao *et al* 2005; Tait & Sutherland 2002) by one member of the biofilm consortium.

It is known that phages are able to infect and lyse cells found on single species communities (Curtin & Donlan 2006; Hanlon *et al* 2001; Hughes *et al* 1998; Lu & Collins 2007; Sharma *et al* 2005; Sillankorva *et al* 2004; Sillankorva *et al* 2008a; Tait *et al* 2002). However, to the authors' knowledge there is only one phage infection study on dual species biofilms (Tait *et al* 2002). In a previous study, it was demonstrated that *Pseudomonas fluorescens* biofilms, commonly found in dairy industries worldwide were successfully infected by the lytic phage ϕ IBB-PF7 (Sillankorva *et al* 2008b). Here the

application of lytic phages to dual species biofilms of *P. fluorescens* and *Staphylococcus lentus* are presented.

S. lentus is a coagulase-negative (CoNS), novobiocin-resistant and oxidase positive bacterial species that belongs to the *S. sciuri* group. This bacterium is a common inhabitant of the physiological skin flora and mucosal surfaces of domestic and wild animals and frequently isolated from different food products of animal origin. *S. lentus* is not considered pathogenic, however their clinical significance is increasing (Couto *et al* 2000; Dakic *et al* 2005; Johnson *et al* 1991; Marsou *et al* 1999; Monday & Bohach 1999; Omoe *et al* 2002) and the main reason for this rate of increase is the spreading antibiotic resistance among CoNS staphylococci (Otto *et al* 2004).

This work investigated the use of phages for the reduction of single and dual species biofilms from stainless steel slides. The main objectives of this study was to evaluate the capability of phages to reach and infect the hosts, study phage biofilm interaction in the presence of a non-susceptible host, and the ability of phages in decreasing the amount of viable cells from biofilms. It was also intended to study the effect of dynamic versus static phage infection conditions in the overall biofilm biomass removal. Both *P. fluorescens* and *S. lentus* strains used to produce single and dual species biofilms were isolated from a dairy plant after the equipment routine cleaning procedures. Firstly, phage treatment was carried out in single species biofilms. Afterwards, the conditions for the formation of dual species model biofilms were established and finally it was considered interesting to see how the dual species biofilms would respond to phage infection. Initially, dual species biofilms were challenged with a phage cocktail containing two phages, being each specific for one of the hosts. Afterwards, the reduction of *P. fluorescens*, the far more problematic bacterium in a number of food related industries, was attempted with phage ϕ IBB-PF7A alone as the effective controlling reagent.

Results

The effect of biofilm formation conditions in single species biofilms

Single species biofilms of *P. fluorescens* and *S. lentus* formed under different conditions were characterized in order to evaluate the effect of biofilm formation conditions in biofilm growth. These biofilms were formed on stainless steel slides during 24 or 72 h under dynamic conditions (continuous shaking at 100 rpm) without renewal of nutrient broth medium (DNR), or with renewal of medium every 12 h (DR), or under static conditions (without shaking) with renewal of medium (SR). The number of viable cells on single species biofilms of *P. fluorescens* and *S. lentus* and total viable cells on dual species biofilms, formed under different conditions, as well as the percentage of *P. fluorescens* in dual species biofilms are presented in Table 3.2.

Table 3.2 Mean number of cells present in 24 and 72 hours old single and dual species (DS) biofilms of *P. fluorescens* and *S. lentus* formed under different conditions and percentage of *P. fluorescens* on dual species biofilms.

Biofilm formation			Mean cell density [$\log_{10}\text{CFU}_i \text{ cm}^{-2} (\pm \text{SD})$]			<i>P. fluorescens</i> in DS (%)	
Shake	Media renewal	Age (h)	Single species		DS		
			<i>P. fluorescens</i>	<i>S. lentus</i>	Total		
DR	+	+	24	4.37 (0.53)	6.35 (0.04)	6.56 (0.08)	32.08 (0.87)
			72	6.41 (0.64)	8.08 (0.13)	8.61 (0.12)	18.33 (4.07)
DNR	+	-	24	3.65 (0.01)	6.34 (0.14)	6.52 (0.03)	23.13 (0.87)
			72	5.80 (0.12)	7.17 (0.25)	7.64 (0.38)	37.50 (3.90)
SR	-	+	24	5.82 (0.19)	6.85 (0.10)	6.84 (0.08)	53.40 (3.41)
			72	6.74 (0.06)	8.01 (0.25)	7.00 (0.16)	51.14 (3.83)

SD means standard deviation of 6 parallel measurements, DR dynamic biofilm with renewal of media every 12 h, DNR dynamic biofilm with no renewal of media, SR static biofilm with renewal of media every 12 h

S. lentus biofilms showed a slightly faster growth, after 24 hours, when cultivated under static compared to dynamic conditions. Nonetheless, after 72 hours there was no significant difference between shaken and non-shaken cultures. However, biofilm cell density in *S. lentus* biofilms was clearly dependent on nutrient supply and an approximately 10-fold higher cell number was detected in 72 hours biofilms when the medium was exchanged. Under static conditions, *P. fluorescens* biofilms formed for 24

hours contained approximately 28 and 148 times more cells than the two distinct dynamic biofilms, DR and DNR respectively. Like in *S. lentus* studies, the biofilms of *P. fluorescens* formed under non-renewal medium conditions had noticeably fewer cells in comparison to biofilms which experienced regular medium exchange. Furthermore, *S. lentus* succeeded better to colonize the metal surface compared to *P. fluorescens*, with overall 10 to 1000 times more cells detected (Table 3.2).

This study shows that the biofilm formation conditions influence differently the number of viable cells of *S. lentus* and *P. fluorescens* present on the substratum and was highest for both single species biofilms with static incubation and medium renewal.

Single species biofilms infected with phages

Phages are able to infect biofilm cells. The *S. lentus* phage ϕ IBB-SL58B and *P. fluorescens* phage ϕ IBB-PF7A, previously characterized in Chapter 2, were used in the biofilm infection experiments. Single species biofilms of *S. lentus* and *P. fluorescens* were infected with phages ϕ IBB-SL58B and ϕ IBB-PF7A and the amounts of viable cells and phages present in the planktonic phase and those adsorbed to the SS substratum and remaining biofilm cells were enumerated (Table 3.3). So far no studies have been performed with phages specific for *S. lentus*. Therefore, in analogy to the treatment of different single species biofilms of *P. fluorescens* with the phage ϕ IBB-PF7A infection experiments (sub-chapter 3.1), it was interesting to study the effect of phage ϕ IBB-SL58B, on the above described single species biofilms of *S. lentus*.

In general, cell decrease by phages was highly related with the amount of cells present in the distinct biofilms. Under all studied situations, cell reduction was approximately 10 to 100 fold higher in 72 hours old biofilms compared to 24 hours old biofilms (Table 3.3).

The best cell reduction in *P. fluorescens* biofilms was obtained without shaking and with repeated medium renewal (SR) where approximately a 3 magnitude reduction was observed in both 24 and 72 hours old biofilms. Compared to infection experiments with the *P. fluorescens* phage, under SR conditions phage ϕ IBB-SL58B had a lower lytic efficiency and the cell removal was approximately 100 fold less efficient in the infection of *S. lentus* biofilms (Table 3.3).

Table 3.3 Biofilm cell reduction and number of planktonic and adsorbed phages after 4 h of phage ϕ IBB-PF7A or ϕ IBB-SL58B infection of *P. fluorescens* and *S. lentus* biofilms, respectively.

Biofilm	Age (h)	Cell reduction		Number of phages after 4 h			
		[log ₁₀ (CFU cm ⁻²) (± SD)]		[log ₁₀ (PFU ml ⁻¹) (± SD)]			
		<i>P. fluorescens</i>	<i>S. lentus</i>	ϕ IBB-PF7A		ϕ IBB-SL58B	
				Planktonic	Adsorbed	Planktonic	Adsorbed
DR	24	2.11 (0.10)	1.56 (0.12)	8.70 (0.19)	4.76 (0.31)	9.07 (0.03)	5.72 (0.37)
	72	2.90 (0.41)	3.36 (0.14)	9.33 (0.05)	5.21 (0.05)	9.63 (0.22)	6.29 (0.17)
DNR	24	1.05 (0.13)	2.58 (0.28)	7.64 (0.02)	5.51 (0.18)	9.04 (0.23)	5.43 (0.21)
	72	2.90 (0.23)	3.59 (0.31)	8.59 (0.23)	5.69 (0.39)	9.82 (0.23)	6.38 (0.02)
SR	24	2.98 (0.65)	1.12 (0.14)	8.78 (0.01)	7.06 (0.49)	8.19 (0.07)	4.95 (0.52)
	72	3.23 (0.48)	2.16 (0.15)	8.78 (0.09)	6.99 (0.35)	9.37 (0.37)	4.30 (0.01)

SD means standard deviation of 6 parallel measurements, DR dynamic biofilm with renewal of media every 12 h, DNR dynamic biofilm with no renewal of media, SR static biofilm with renewal of media every 12 h

When *S. lentus* biofilms were formed under shaking conditions, with (DR) and without medium renewal (DNR) respectively, the initial number of cells present on the substratum at the time of infection was higher (Table 3.2) and, interestingly, also cell lysis was significantly higher with the ϕ IBB-SL58B phage and *S. lentus* biofilms, compared to phage ϕ IBB-PF7A infection of *P. fluorescens* biofilm cells (Table 3.3).

In summary, the main difference observed in the experiments with phage infection of single species biofilms was the very efficient reduction by ϕ IBB-SL58B of *S. lentus* biofilms under shaking and without medium renewal (DNR) compared to *P. fluorescens* biofilms infected with ϕ IBB-PF7A (Table 3.3). In contrast, under static conditions, a poor cell lysis of *S. lentus* was observed and the number of PFUs was the lowest. The experiments also showed that, even if the efficiency of lysis was low within the investigated time window, ϕ IBB-SL58B adsorbed well to the SS slides and to the remaining cells present on the surfaces (Table 3.3).

The infection of both single species biofilms with their respective phages was quite efficient and resulted in a relatively low number of survivors, 1×10^3 cells ml⁻¹ for 24 hours old SR *P. fluorescens* biofilms and 1×10^4 cells ml⁻¹ for 24 hours old DNR *S. lentus* biofilms which were observed to be the best infection conditions, respectively (Table 3.3).

The data of this study show clearly that the hydrodynamic and medium renewal conditions during biofilm formation and phage treatment must be considered and may vary for different phage/host systems. ϕ IBB-SL58B showed an enhanced ability of infecting *S. lentus* under shaking conditions without medium removal (DNR), while ϕ IBB-PF7A attacked *P. fluorescens* biofilms most efficiently under static conditions.

Characterization of dual species biofilms

P. fluorescens and *S. lentus* were used to produce dual species biofilms using the biofilm formation methods above described for single species biofilms. Total cell counts and the percentage of *P. fluorescens* cells present on dual species biofilms are presented in Table 3.2. Also, confocal laser scanning microscopy (CLSM) was used to verify the overall coverage of the stainless steel slides by cells using the three different biofilm formation conditions (DR, DNR and SR) and incubation for 24 and 72 hours (Figure 3.7).

Interestingly, for 24 hours old dual species biofilms neither medium renewal nor shaking did influence much the amount of cells (Table 3.2). However, these 24 hours old biofilms formed under DNR and SR consisted of a sparse single layer of cells with a small number of cell clusters while the biofilms formed under DR conditions of a greater number of cell clusters and aggregates (Figure 3.7). The biofilm architecture was clearly more developed after 72 hours and there was an increase of the size and number of cell clusters in the different biofilms. The highest number of cells in 72 hours old dual species biofilms was observed under dynamic and medium renewed conditions (Table 3.2) where biofilms presented a thicker structure, as observed on the orthogonal sections showing the side (xz and yz) views, and covered the stainless steel slides entirely (Figure 3.7). Also, contrarily to 72 hours old single species biofilms of *S. lentus* and *P. fluorescens*, which exhibited the highest cell number when formed under static conditions, prolonging the formation of dual species biofilms to 72 hours using static conditions resulted in the smallest cell amount, clearly visible by CLSM (Figure 3.7). As shown by viable cell counts, both 24 and 72 hours old biofilms formed under the two studied dynamic conditions, DR and DNR respectively, were predominantly formed by *S. lentus* cells and the percentage of *P. fluorescens* was always below 40% and was the lowest for 72 hours old DR biofilms. Contrarily to these two types of biofilms, the ones formed without shaking (SR) had approximately equal amounts of *P. fluorescens* and *S. lentus* cells (Table 3.2).

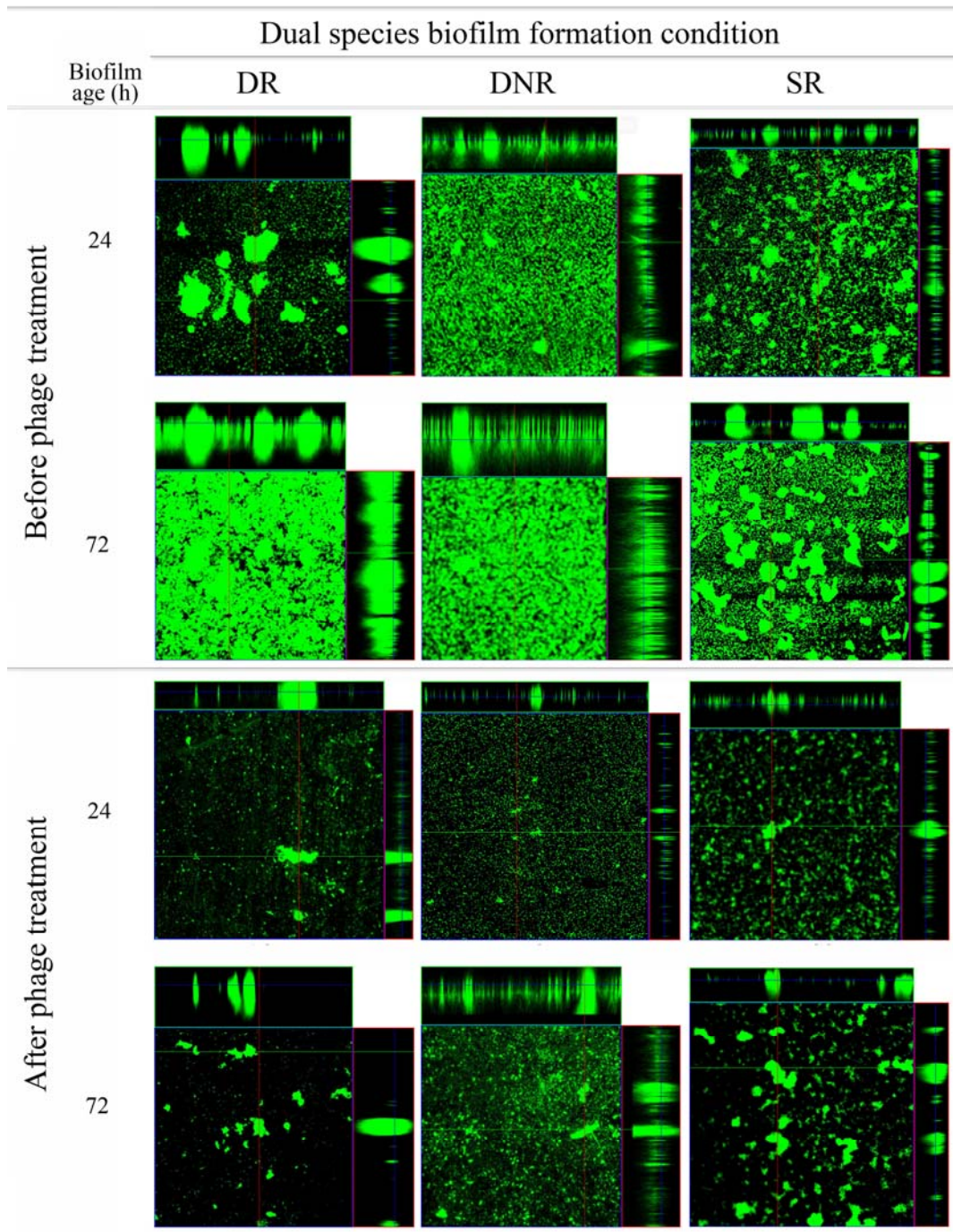


Figure 3.7 – Confocal laser scanning micrographs (20 × magnification) of the overall coverage of stainless steel slides with dual species biofilms. Biofilms were formed under dynamic media renewal (DR), dynamic non-media renewal (DNR) and static media renewal (SR) conditions for 24 and 72 hours and afterwards infected using a cocktail of phages ϕ IBB-PF7A and ϕ IBB-SL58B. Shown are biofilms stained with DAPI before and after 4 hours of infection with the phage cocktail. Orthogonal sections show side views (xz and yz) of the biofilm z-stack images at the selected points.

In summary, it was observed that the conditions to produce the highest cell counts were different for single species *S. lentus* and *P. fluorescens* biofilms compared to experiments where the two bacteria co-existed in a same substratum.

Dual species biofilms challenged with phages ϕ IBB-PF7A and a phage cocktail

After establishing the conditions for the formation of dual species biofilms, it was then studied how dual species biofilms would respond to phage infection.

Firstly, dual species biofilm control was performed with a cocktail of phages ϕ IBB-PF7A and ϕ IBB-SL58B specific for *P. fluorescens* and *S. lentus* respectively. Phage infection was evaluated by viable counts of the total number of cells (counted as CFU) present in the biofilms and in the planktonic phases (Figure 3.8). Also, phage replication was assessed by the number of phages adsorbed to the coupons and those existing at the planktonic phase number of phages adsorbed to the coupons and those existing at the planktonic phase (Figure 3.9).

The application of the phage cocktail to all different types of biofilms resulted in cell reductions compared to the biofilm controls, where the stainless steel coupons were immersed for 4 hours in a solution of saline and TSB (1:1 v/v). The phage cocktail infected best the biofilms formed without medium renewal and worse the ones formed without agitation. Although a higher initial cell number was observed in 72 hours old biofilms compared to the 24 hours old biofilms, phages were able to reduce both biofilms to the same level of viable cells present on the coupons and thus higher cell decreases were observed in infected 72 hours old biofilms (Figures 3.8). Furthermore, in the phage-biofilm interaction studies performed, 2 hours of biofilm treatment with phages was enough to achieve a good cell lysis and prolonging the phage treatment to 4 hours only slightly decreased the amount of cells present on biofilms. CLSM micrographs, taken after 4 hours of phage exposure, show a considerable reduction of cells on the substratum when the treatment was carried out under shaken conditions and they also confirmed that the treatment was less efficient under static conditions (Figure 3.7). Although the cell reduction observed under shaken conditions was remarkable, phage treatment always left a number of surviving cells (Figure 3.8).

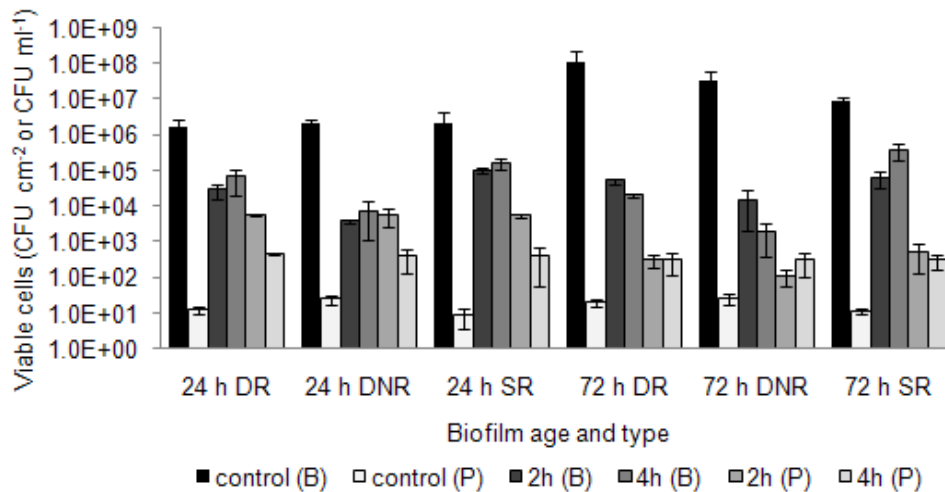


Figure 3.8 – Number of cells present on biofilms (B) (CFU cm⁻²) or planktonic phase (P) (CFU ml⁻¹) after 2 and 4 hours of infection of 24 and 72 hours old dual species biofilms *P. fluorescens* and *S. lentus* using a cocktail of phages ϕ IBB-PF7A and ϕ IBB-SL58B. Phage infected biofilms were formed under dynamic media renewal (DR), dynamic non media renewal (DNR) and static media renewal (SR) conditions and total viable cell counts were performed. Controls were 4 hours in saline-TSB solution and biofilm cells and planktonic phase cells were enumerated. For all experiments n = 6. The error bars indicate standard deviations.

Control experiments carried out with the different types of biofilms, during 4 hours in saline-TSB, showed always a low cell release from the biofilms to the planktonic phase (< 30 CFU ml⁻¹). Contrarily to control experiments, the application of phages induced a considerable release of cells to the planktonic phase and more than 10³ CFU ml⁻¹ were always detected (Figure 3.8). The only advantage of prolonging the infection period from 2 to 4 hours was that it allowed phages to further decrease the planktonic cell numbers (Figure 3.8). Furthermore, the higher cell amounts detected in 72 hours old biofilms resulted in a much lower planktonic cell numbers than those detected after phage infection of 24 hours old biofilms (Figure 3.8). This was due to a somewhat higher quantity of phages present due to lysis of more cells (Figure 3.9).

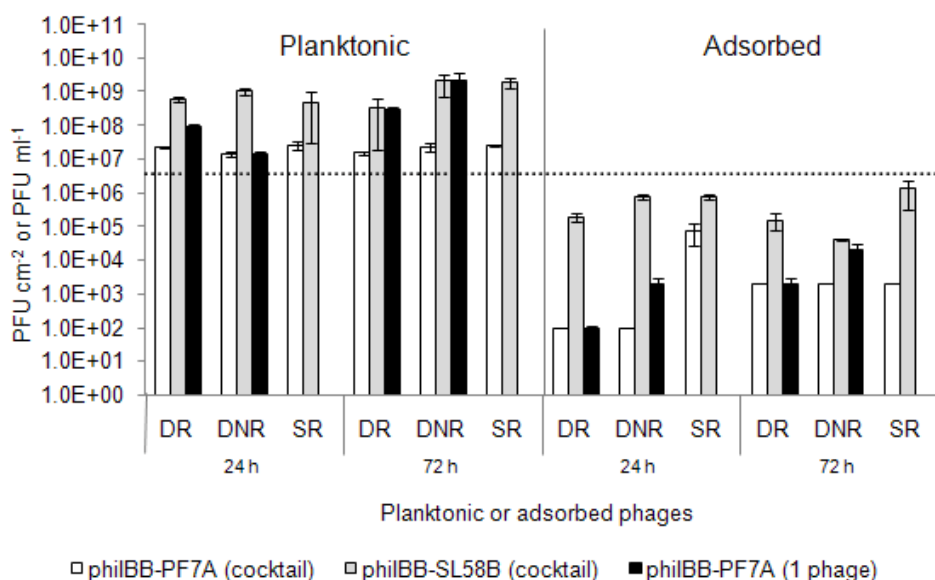


Figure 3.9 – Number of phages on the planktonic medium (PFU ml⁻¹) or adsorbed to the stainless steel slide and biofilms (PFU cm⁻²) after 4 h of phage infection of different dual species biofilms. Dual species 24 h and 72 h biofilms of *P. fluorescens* and *S. lentus* were infected with: a phage cocktail or only with phage ϕ IBB-PF7A. Horizontal line represents the number of phages in the beginning of the experiment. For all experiments n = 6. The error bars indicate standard deviations.

In general, the phage numbers obtained after biofilm treatment with the phage cocktail indicate that both phages, ϕ IBB-SL58B and ϕ IBB-PF7A, were able to replicate well in all investigated biofilms (Figure 3.9). High numbers of phages were observed in the planktonic phase. However, the number of progeny phages adsorbed to dual species biofilms and the substrata was lower than observed in single species biofilm infection experiments with the two phages (Table 3.3 and Figure 3.9). In dual species biofilms the adsorbed phage numbers varied between 1×10^2 and 1×10^5 PFU ml⁻¹ (Figure 3.9) while in the single species *P. fluorescens* biofilm studies the numbers were remarkably higher and between 1×10^5 and 1×10^7 PFU ml⁻¹ (Table 3.3).

Comparing single infected with the specific phages and dual species biofilms infected with a phage cocktail, it was clearly visible that the infection followed the kinetic observed in *S. lentus* biofilms infected with phage ϕ IBB-SL58B. This was already expected as the dual species biofilms were predominantly formed by *S. lentus*. Dual species biofilms formed under SR conditions exhibit similar percentages of both bacteria and although single species biofilms were well reduced under non-shaken conditions, with 100 and 1000 fold reduction of *S. lentus* and *P. fluorescens* cell counts in 72 hours

old biofilms, the decrease detected in dual species was clearly diminished (Table 3.3 and Figure 3.8).

After studying the influence of a phage cocktail to dual species biofilms and having observed that it could greatly reduce the amount of *P. fluorescens* and *S. lentus* cells in the biofilms, a next experiment was performed applying only one phage specific for the less predominant bacterium present in the biofilms, *P. fluorescens* (cf. Table 3.2). Although *P. fluorescens* phage performed best in single species biofilms formed under static conditions (Table 3.3), the application of a phage cocktail to dual species showed to be inefficient (Figure 3.8) and therefore static experiments were not carried out. Thus, *P. fluorescens* viable cells in the dual species DR and DNR biofilms and total cells released to the planktonic media were enumerated after 4 hours of infection (Figure 3.10).

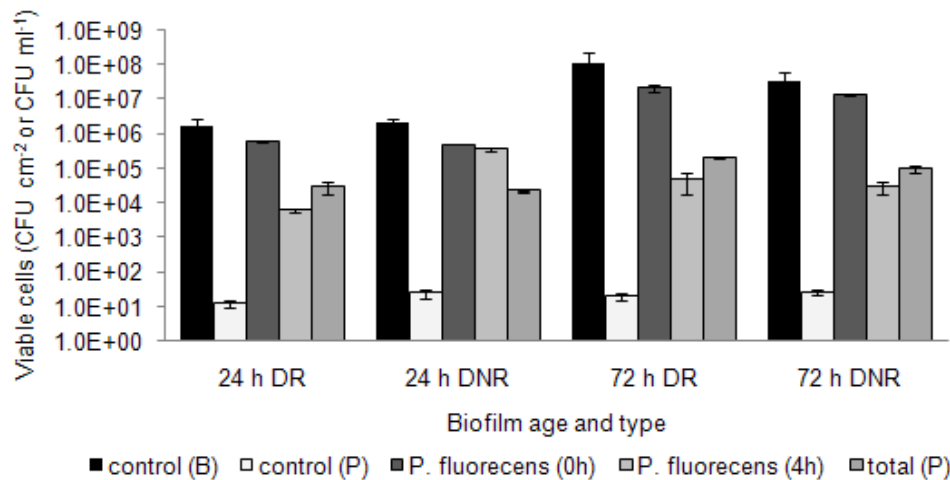


Figure 3.10 – Number of cells present on biofilms (B) (CFU cm⁻²) or planktonic phase (P) (CFU ml⁻¹) after 4 hours of infection of 24 and 72 hours old dual species biofilms of *P. fluorescens* and *S. lentus* using phages ϕ IBB-PF7A. Phage infected biofilms were formed under dynamic media renewal (DR) and dynamic non media renewal (DNR) and *P. fluorescens* cells on biofilms and total viable cell counts of cells in the planktonic phase were performed. Controls were 4 hours in saline-TSB solution and biofilm cells and planktonic phase cells were enumerated. For all experiments n = 6. The error bars indicate standard deviations.

Assessing the numbers of *P. fluorescens* cells on the different biofilms, it became obvious that phage ϕ IBB-PF7A can successfully reach its host. With the exceptions of 24 hours old DNR biofilms, cell reduction from the biofilms was confirmed in all other experiments carried out. Contrarily to the practically inexistent decrease detected with 24 hours DNR biofilms, the cell amount from 72 hours old DNR biofilms was decreased by two orders of magnitude (Figure 3.10). Also, an interesting difference was observed in the

number of cells present in the planktonic phase. Dual species biofilm infection with ϕ IBB-PF7A alone resulted in a 100-fold increase of the number of cells in the planktonic phase compared to the treatments with a phage cocktail (cf. Figures 3.8 and 3.10). It may be assumed that, when only ϕ IBB-PF7A was applied, *S. lentus*, the non-susceptible bacterium present and predominant in the DR and DNR dual species biofilms (Table 3.2), detaches from the infected biofilms. Furthermore, a more detailed analysis of the phage numbers shows that phage ϕ IBB-PF7A can better reach the host when applied alone as there was observed an increase of phage numbers on the planktonic phase and adsorbed to the remaining biofilms present on the substrata compared to experiments with biofilm treatment with the phage cocktail (Figure 3.9).

In summary, the application of a phage cocktail with phages specific for each host present in a dual species biofilm reduced not only the number of bacteria present in the biofilms but also kept the cell numbers of bacteria in the planktonic phase at a low level. Moreover, a phage, specific for one of the hosts of a dual species biofilm, can successfully reach the host and lead to an efficient cell reduction under dynamic conditions.

Discussion

In this work, phage control of dual species biofilms of two bacteria that co-exist in dairy plants was studied. This is the first study where biofilms formed by Gram-positive (*Staphylococcus lentus*) and Gram-negative bacteria (*Pseudomonas fluorescens*) were subjected to lytic phages. Phage control of such biofilms was investigated by two approaches, with phages specific for either one or both of the hosts, in order to understand what occurs to the biofilms in these two different scenarios.

This experimental work indicates that a single phage applied to a dual species biofilm can efficiently reach the host and reduce their cell number in the biofilm, but also may cause the release of the non-susceptible species to the planktonic phase. Furthermore, application of a phage cocktail for each of the hosts of the dual species biofilm decreased not only the cell number in the biofilm, but also the cells which were released to the planktonic phase. Moreover, the conditions under which the phages are applied are important factors to be considered.

Biofilm destruction and cell lysis were efficient in single species biofilms formed under non-shaken conditions, however phages were incapable of decreasing the bacterial hosts when mixed in a dual species biofilm. Thus, under non-shaken conditions, *S. lentus* and *P. fluorescens* cells appear to be protected from phage infection. The results of this work are in agreement with the work of Tait and colleagues that have suggested that the presence of a non-susceptible bacterial population within a biofilm can protect phage-susceptible bacteria from being attacked by the phage (Tait *et al* 2002). However, it cannot be assumed that bacterial protection takes always place as evidenced by the great cell decreases obtained after phage infection of DR and DNR biofilms, respectively. However, the reason for a less successful decrease under static conditions, after 4 hours of phage exposure, also suggests a possible inefficient phage-host interaction. Another possible explanation, for the low phage infection efficacy in static biofilms can be a possibly higher polysaccharide and protein content than in dynamic biofilms. It is known that biofilms formed under distinct hydrodynamic conditions exhibit different amounts of cellular and extracellular polysaccharides and proteins. Simões *et al.* showed that biofilms of *P. fluorescens* ATCC 13525^T formed under laminar flow conditions have a much higher content of cellular and extracellular polysaccharides and proteins than turbulent flow-generated biofilms. However, this explanation for the different cell lysis detected in shaken and non-shaken biofilms needs further investigation to be proved.

The addition of a single phage or the two phage cocktail to dynamic dual species biofilms resulted in a 1000 fold cell reduction of 72 h old dual species biofilms within only 2 to 4 hours. This efficiency is comparable with other reported studies of biofilms infected with phages over a 24 hour period (Hanlon *et al* 2001; Lu & Collins 2007; Sharma *et al* 2005; Tait *et al* 2002). In this work, 2 hours of biofilm exposure to phages lead to a great reduction of the biofilm and prolonging the exposure period to 4 hours slightly enhanced the cell reduction. The results of this work are in agreement with the ones obtained by Hughes *et al.* (1998) where the maximum reduction of viable cells in biofilms was obtained already within 2 h or 5 h, depending on the biofilm studied, after which there was no further decrease. These results may indicate that prolonged phage infection periods are not required and short application periods may even be beneficial to avoid resistance acquisition by the hosts.

The use of phages to treat and control established single species biofilms of *P. fluorescens* and *S. lentus* indicated the principal possibility of obtaining efficient reduction of the biofilm cell numbers. *P. fluorescens* biofilms are well infected under

static conditions while under these conditions *S. lentus* biofilms are inefficiently attacked. Phage treatment of *P. fluorescens* biofilms was inefficient when the biofilm was grown without renewal of medium, i.e. if biofilms experienced severe starvation. In contrast and surprisingly, *S. lentus* biofilms were best removed under severe starvation. This finding is especially interesting, as it is well known that phage development depends on the physiological state of the host which directly reflects on the intracellular resources available for phage reproduction (Hadas *et al* 1997). Therefore, the result obtained in this work was unexpected as starvation state cells commonly would be suggested to result in a decreased number of progeny phages and longer phage reproduction cycles (Los *et al* 2007; Neubauer *et al* 2006). The efficiency of biofilm removal was well in accordance with different burst sizes of the two phages, 507 PFU per infected cell for ϕ IBB-SL58B and 153 PFU per infected cell for ϕ IBB-PF7A, respectively, as characterized in Chapter 2. Furthermore, this reveals the importance and challenge of performing a careful selection of phages for the use in multi-species biofilms.

This study shows the importance of selecting a proper model system for the development of biofilm control studies with phages. Factors such as mixing and medium supply can influence the infection process however they may be optimized to obtain an efficient biofilm control. It also became clear that other species in a consortium affect the treatment. Nevertheless, this investigation proves that both single and dual species biofilms can be efficiently controlled by phages and despite the presence of a non-susceptible host, phages can successfully reach and lyse the target host bacterium. However, for technical applications the effect of the biological control may be enhanced by other methods, such as the use of chemical agents to obtain biofilm eradication. The potential of such a combination seems to be attractive, as the results described in this work indicate that even the use of a single phage weakens the biofilm and leads to a release of non-attacked bacteria to the medium, where they could be efficiently killed. Also, it seems likely that the total amount of chemical agents used for biofilm control could be significantly reduced if the biofilm is already partially disintegrated by phages.

Materials and Methods

Bacteria, bacteriophage and growth conditions

Pseudomonas fluorescens and *Staphylococcus lentus* were isolated from a dairy plant (Paços de Ferreira, Portugal). Both bacteria were grown at 30 °C in Tryptic Soy Broth (TSB, Fluka) or in solid TSA medium that contained 1.2 % w/v of Bacto™ agar (Difco). The strains were subcultured once and glycerol stocks were generated and stored frozen at –80 °C until further use. Bacteriophages ϕ IBB-PF7A and ϕ IBB-SL58B were isolated from raw sewage (wastewater treatment plant, Esposende, Portugal) and purified and concentrated using the double soft agar layer (Sambrook & Russell 2001) where the soft agar top-layer of TSB contained 0.6 % of Bacto™ agar.

Phage titration of stock solution

Bacteriophage ϕ IBB-PF7A and ϕ IBB-SL58B titers, were analyzed as described by Adams (Adams 1959).

Biofilm formation

Single and dual species biofilms of *S. lentus* and of *P. fluorescens* were formed on stainless steel (SS) 1 × 1 cm slides, immersed in 6-well microplates with 6 ml of TSB medium, during 24 h and 72 h under dynamic media renewed (DR), dynamic media non-renewed (DNR) and static media renewed (SR) conditions according to the method described in sub-chapter 3.1.

Biofilm infection

Biofilms were infected with phage as described previously in sub-chapter 3.1.

CFU and PFU counts of attached or planktonic bacteria or phages

The amount of bacteria present on the SS slides before and after infection of pre-grown biofilms and bacteria released to the planktonic phase after phage infection were enumerated as described by Sillankorva *et al.* (2008b) in order to estimate the efficiency of phages in lysing the cells. Briefly, SS slides with biofilms before phage infection were washed by immersion in PBS and inserted in 50 ml tubes with 6 ml of saline (0.9 % NaCl). The tubes were vigorously vortexed (4 × 30 sec) and the SS slides were

immediately removed to prevent bacterial reattachment. Afterwards, dilutions were made in saline for CFU counts and plated on TSA Petri dishes, or made in SM buffer, and mixed with the host bacterium and soft-agar and poured onto TSA Petri dishes. For CFU or PFU counts of planktonic bacteria or phages, samples were diluted in saline or SM buffer and equally plated. In order to verify if SM buffer would interfere with the bacterial release from biofilms, control experiments were performed where SS slides with biofilms were washed by immersion in PBS and inserted in 6-well microplates with 3 ml of SM buffer and 3 ml of TSB and the microplates were subjected to the same conditions as those performed in phage infection experiments. Afterwards, these control SS slides were once again washed by immersion in PBS and inserted in 50 ml tubes with 6 ml of saline, vortexed as described above and plated. For *P. fluorescens* cell counts, the selective medium *Pseudomonas* Isolation Agar (PIA) (Sigma-Aldrich, St. Louis, MO) was used and the procedure was the same as adopted for total cell counts. The Petri dishes were incubated at 30 °C for 24 h and colonies were counted. Six independent parallels were performed for the attached and planktonic CFU and PFU countings.

Microscopy and image processing

Image acquisition was performed using a Zeiss LSM 5 PASCAL confocal laser scanning microscope (CLSM) (Carl Zeiss MicroImaging GmbH, Jena, Germany). Stainless steel (SS) slides with biofilms for CLSM were rinsed by immersion in PBS and immersed into a 2.5% glutaraldehyde solution (4°C, 1h) for fixation. Afterwards, the SS slides were rinsed with PBS and stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) (Molecular Probes, Carlsbad, CA, U.S.A) according to the manufacturer's instructions. The LSM image browser software was used for analysis of biofilm images and z-stacks were acquired from random positions through the biofilm avoiding the edges of the SS slides.

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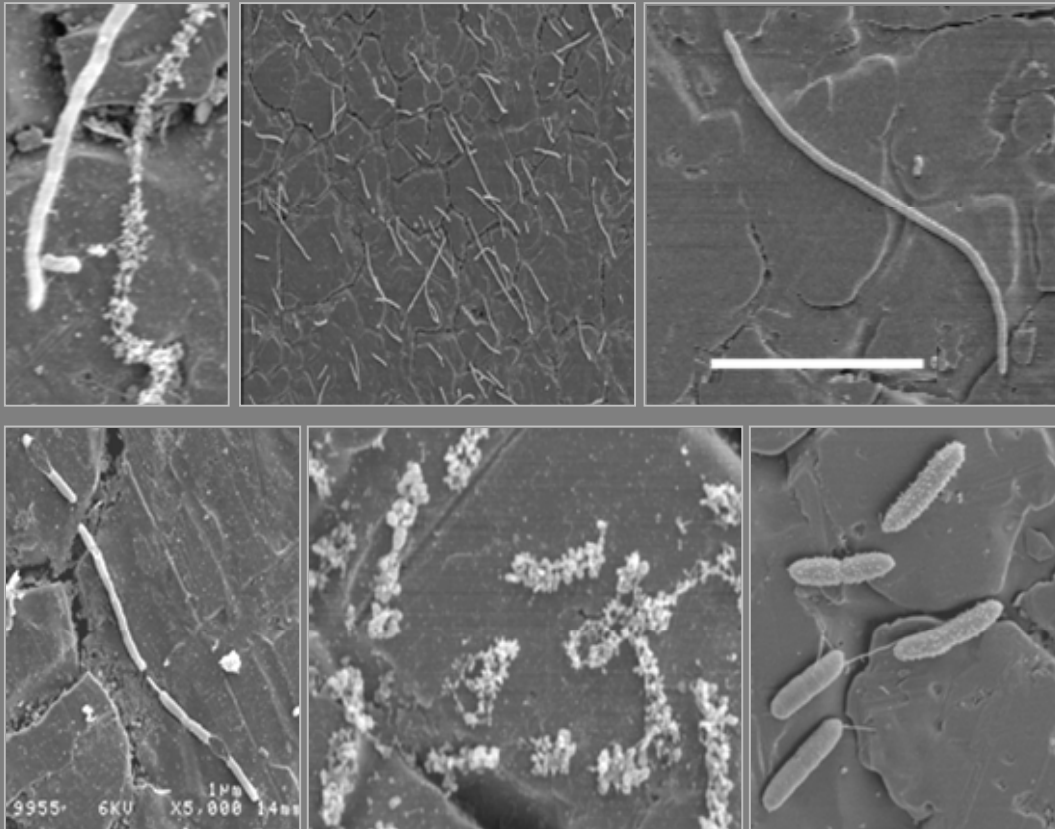
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1. LITERATURE REVIEW
2. ISOLATION AND CHARACTERIZATION OF PHAGES
3. PHAGE BIOFILM INTERACTION

4. PHAGE AND PLANKTONIC CELLS

5. MAIN CONCLUSIONS AND SUGGESTIONS FOR FORTHCOMING WORK



4.1. Action of the lytic phage ϕ IBB-PF7A on rod-shaped and filamentous-like *P. fluorescens* cells

Abstract

Aims: Occasionally bacteria suffer changes acquiring uncommon morphologies. These changes can cause problems for phages to infect their hosts. This work describes the morphological changes occurring on *Pseudomonas fluorescens* cells due to exposure to a variety of temperatures and rotary agitations and investigates the efficacy of a lytic phage in controlling rod and filamentous shaped hosts.

Methods and Results: *P. fluorescens* cell morphology changed from rod to filamentous shaped above speeds of 100 rpm and 25°C. Low temperatures and no agitation delayed the beginning of lysis and increased the period of cell lysis. Furthermore, phage acted fastest on mid-logarithmic cells and early stationary cells were lysed after a brief regrowth period. Nevertheless, phages were able to infect particularly well the stationary cells and high density decrease percentages were achieved. Phage reproduction showed to be dependent on two factors: cells growing at a faster rate were capable of producing more phages and lengthened cells were able to produce high phage progeny inside the hosts.

Conclusions: *P. fluorescens* cell morphology showed to be highly influenced by temperature and rotary agitation. Phage ϕ IBB-PF7A infection of rod and filamentous cells resulted, in general, in high cell density reductions.

Significance and Impact of the Study: This is the first study carried out with a lytic phage capable of infecting planktonic *P. fluorescens* cells with different morphologies and lengths. Furthermore, this phage was able to target and reproduce inside cells at different growth phases including late-logarithmic and stationary phase cells.

Introduction

Bacteriophages have for quite some time been suggested as good biocontrol alternatives to the traditional agents used in a variety of environments (Atterbury *et al.* 2003; Breeuwer *et al.* 2005; Curtin & Donlan 2006; Goode *et al.* 2003; Loessner & Carlton 2005; Withey *et al.* 2005) possessing many advantages over chemical agents. Firstly, phages have a high specificity against a host or host range which implies a safe and targeted elimination without altering the surrounding microflora. Also, phages are readily present in all environments, although not at the necessary concentration for being considered biocontrol agents, and are utterly biodegradable (UV, temperature, etc.) and therefore functional in sustainable, environmental-friendly systems. Ultimately, phages reproduce when the target bacterium is present increasing their concentration in the respective environment, through reproduction inside the host cells.

A variety of environmental alterations may result in morphological and physiological changes on the host bacterium that include cell volume decrease, cell shape change, nucleoid compactation, alteration of cell wall composition, accumulation of some storage material, etc. (Huisman *et al.* 1996; Ishihama 1999; Kolter *et al.* 1993; Roszak & Colwell 1987). Consequently, these cell alterations may influence the phage reproduction and infection process. The effectiveness of a phage in lysing the respective host bacterium depends greatly of the target bacterium availability and density but also of factors that cause stress on microbial cells such as: nutrient concentration, temperature, life history strategies (De Paepe & Taddei 2006; Moebus 1996; Sillankorva *et al.* 2004). In a previous study with a dairy *P. fluorescens* isolate, biofilms of this host exhibited either rod or filamentous morphology depending on the biofilm formation conditions used (Sillankorva *et al.* 2008). In this study, morphological changes in planktonic cells were induced by exposing the cells to different: (i) rotary agitations and (ii) temperatures in order to study phage performance against different cell shapes and physiologies.

Results

In a previous study, *P. fluorescens* biofilm cells were shown to acquire a filamentous morphology when the formation occurred under the effect of two conditions: rotary agitation at 100 rpm and at 30 °C (sub-chapter 3.1). The work described here investigates the effect of temperature (5 - 30 °C) and rotary agitation (0 – 200 rpm) on the length of planktonic cells. Moreover, phage control of cells at different growth phases will be studied.

Effect of temperature

P. fluorescens is a gram-negative bacterium able to grow at temperatures ranging from 4 - 32°C. In order to assess the effect of temperature in cell morphology, cultures were grown from 5 to 30 °C, until reaching mid-exponential phase and the cells were visualized by field emission scanning microscopy (FESEM) (Figure 4.1). Also, control of cells using phage solutions with different concentrations (10^7 , 10^8 and 10^9 PFU ml⁻¹) was investigated following the decrease in optical density and phage increase.

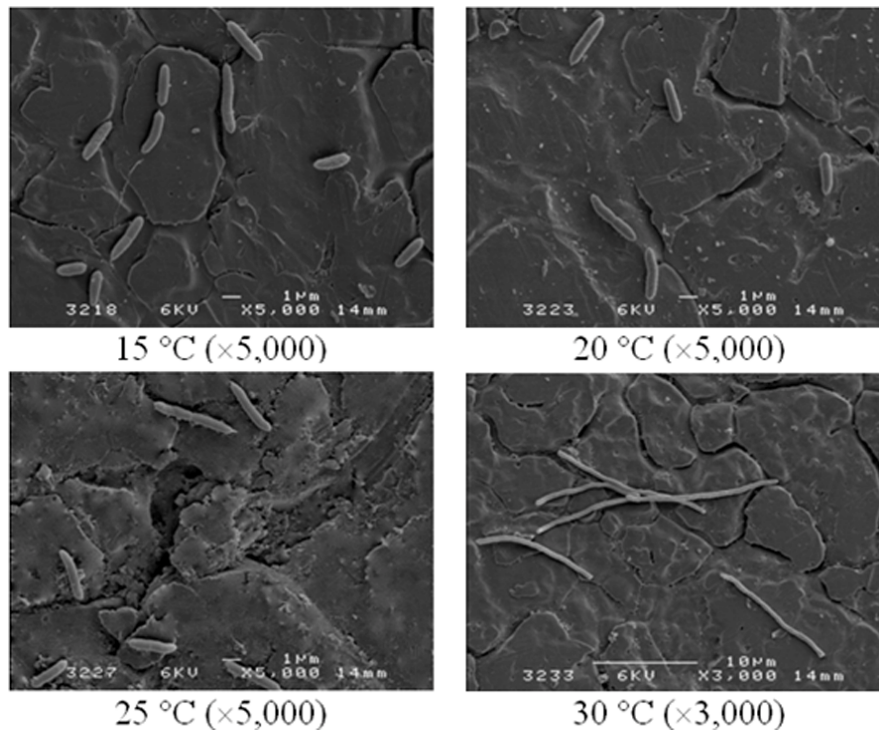


Figure 4.1 - Morphology of *P. fluorescens* cells grown at 160 rpm under varied temperatures.

P. fluorescens cells grown between 5 to 25 °C presented typical rod shape, with cell lengths of approximately 2 – 3 µm. The morphology altered at 30 °C and cells acquired a filamentous shape. At this temperature, a variety of lengths, ranging from 4 up to 24 µm were observed (Figure 4.1 and Table 4.1).

Table 4.1 - Influence of temperature on cell size, growth rate and influence of different phage concentrations on phage infection parameters and cell density decrease (%).

T (°C)	Cell size* (µm)	µ (h⁻¹) (± SD)	Log (PFU/ml)	Beginning of infection (min)	Lysis period (min)	Density decrease (%) (± SD)
5	-	0.025 (0.001)	9	240	285	67.80 (3.08)
			8	360	320	72.82 (0.22)
			7	525	415	82.42 (5.02)
10	-	0.047 (0.002)	9	120	300	69.33 (2.75)
			8	180	240	66.44 (6.64)
			7	330	510	79.65 (1.83)
15	1.7–3.8	0.070 (0.004)	9	60	180	61.54 (4.05)
			8	120	180	62.58 (2.93)
			7	160	325	71.93 (1.15)
20	2.3–3.3	0.087 (0.004)	9	40	160	73.08 (3.75)
			8	80	160	75.15 (4.72)
			7	120	160	78.03 (4.27)
25	2.4–3.4	0.122 (0.003)	9	40	100	82.37 (1.94)
			8	60	120	83.06 (0.72)
			7	60	160	84.13 (0.87)
30	4.2–23.8	0.084 (0.003)	9	40	120	79.75 (6.75)
			8	60	120	78.11 (7.96)
			7	100	80	69.67 (7.62)

* minimum and maximum size from measurements of 5-10 cells, SD means standard deviations

Also, temperature affected greatly the specific growth rate, increasing with temperature and reaching a maximum at 25 °C after which the rate decreased slightly (Table 4.1).

A constant optical density can reflect in different cell numbers depending on the morphology a cell possess. Cell lengths in cultures grown from 5 to 25 °C are similar and a density of 1.0 corresponded approximately to 7.6×10^9 CFU ml⁻¹. Low temperatures,

especially 5 and 10 °C, delayed significantly the beginning of phage infection (Figure 4.2a) and increased the lysis period, which corresponds to the time point right before an optical density decrease was observed until the time point when this decrease ends. Consequently, the long lysis periods observed resulted in lower rates of density decrease (Figure 4.2c). Nevertheless, although phages needed, at low temperatures, a more prolonged period to infect the cells, phages reduced rather well the density of the cultures (66 – 83 %). Phage progeny increase after infection of cells grown between 5 to 25 °C showed that low temperatures resulted in less phage progeny, 1000 up to 10000 fold times less compared to the maximum phage concentration which was achieved at 25 °C (Figure 4.2b). This shows that phage progeny was highly dependent of cell growth temperature, most likely due to the different specific growth rates of the host. At higher temperatures, phages start attacking faster the cells. At 25 °C, the highest density decrease rate (Figure 4.2c) was obtained and there was merely a 20 minute difference in the beginning of phage infection after exposure to the different phage concentrations (10^7 , 10^8 or 10^9 PFU ml^{-1}) (Table 4.1).

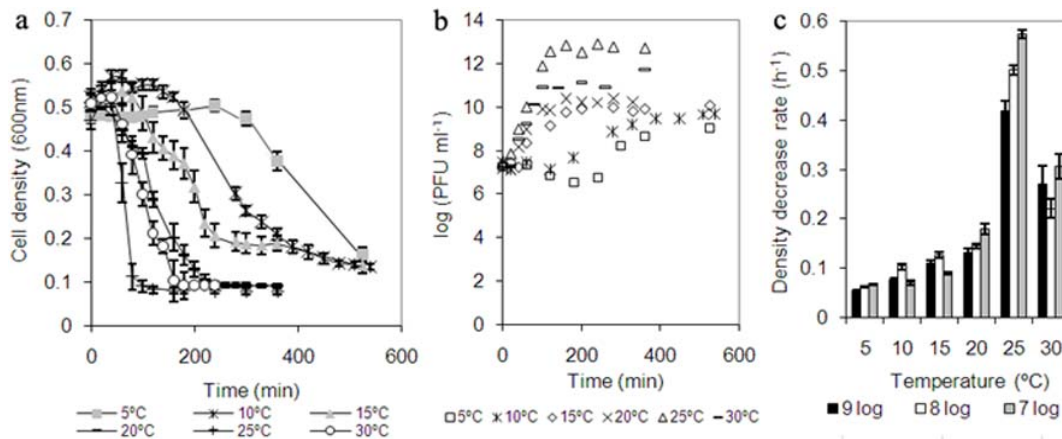


Figure 4.2 – *P. fluorescens* cells grown and infected at different temperatures with phage $\phi\text{IBB-PF7A}$. Measurement of a) cell density; b) phage numbers at different time periods using a phage concentration of 1×10^9 PFU ml^{-1} and 160 rpm and c) cell decrease rates obtained after infection of cells with different phage concentrations. Error bars indicate standard deviations of 24 different wells.

As expected, in all tested temperatures, the use of less concentrated phage solutions lead to longer lysis periods due to the lower number of phages available for a host. Interestingly, the majority of the experiments showed that the use of less concentrated phage solutions resulted in greater density decrease percentages (Table 4.1). This shows

that although the phages required a longer infection period, they were more efficient in eliminating the hosts.

A density of 1.0 (OD at 600 nm) in filamentous cultures (30°C) corresponded to about 5.9×10^8 CFU ml⁻¹. This shows that at 30 °C there was an initial cell concentration 10 times lower than in the experiments carried out at temperatures from 5 to 25 °C however the cells present had a length 10 times longer (Table 4.1). Phage infection at 30 °C was similar to the one observed at 25 °C with comparable periods of beginning of infection and lysis. The main difference besides cell morphology was on the rate of density decrease which was at 30 °C half the value obtained at 25 °C. Phages were able to replicate well inside the filamentous hosts and although there were 10 fold less cells the long length resulted in very high phage progeny at 30 °C (Figure 4.2b).

This study evidences that *P. fluorescens* cell morphology and growth parameters are significantly influenced by temperature. Nonetheless, phage ϕ IBB-PF7A was remarkably efficient lysing both cell morphologies.

Effect of rotary agitation

In a previous study with *P. fluorescens* biofilms it was observed that the biofilm cells formed under static conditions (0 rpm) possessed rod shape while the ones formed under dynamic conditions (100 rpm) had both rod and filamentous shaped cells (Sillankorva *et al.* 2008). In this work it is investigated how different agitations influence the lengths of planktonic cells and observed how these two variables, cell lengths and agitations, influence the phage lytic efficacy. For this purpose, cultures were grown, until reaching a mid-exponential phase, at different rotational agitations (0, 100, 160 or 200 rpm) and maintaining a constant temperature (30 °C). Cells were visualized with FESEM (Figure 4.3), and colony forming units of cells grown at different agitations were performed (Table 4.2).

Like in the experiments with *P. fluorescens* biofilms (Sillankorva *et al.* 2008), planktonic cells grown without any rotary agitation exhibited a rod shape morphology, with minimum and maximum cell lengths of 1.5 and 3.3 μ m respectively. The increase of the agitation speed caused a lengthening of the cells and a change from rod to filamentous morphology (Table 4.2 and Figure 4.3). At the maximum speed studied, the cell lengths were 10 times greater than those registered at 0 rpm. Other parameter highly influenced

by the rotary agitation was the specific growth rate, which increased significantly with the agitation speed applied (Table 4.2).

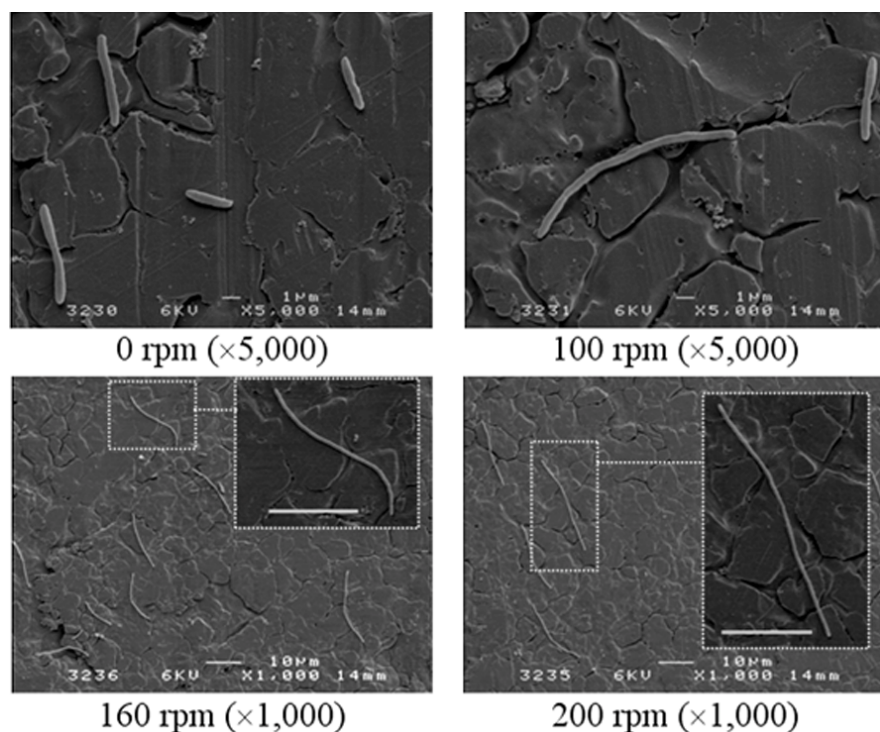


Figure 4.3 – Morphology of *P. fluorescens* cells grown at 30 °C under varied shake speeds.

Contrarily to the effect of temperature, the use of different rotational agitations did not influence greatly the start of cell lysis. However, the lack of agitation (0 rpm) caused a particularly prolonged lysis period, a very low density decrease (Table 4.2), and a reduced rate of density decrease (Figure 4.4b). A density of 1.0 (OD at 600 nm) in both cells suspensions grown at the maximum agitation speed and with no speed corresponded to different cell concentrations, being the filamentous suspension 2.3 log (agitated culture) lower than the rod shape one. Once again the lower colony counts were due to the increase of the cell lengths (Table 4.2).

Cells with varied lengths were well infected at speeds from 100 to 200 rpm. Interestingly, the amount of progeny released after application of 1×10^9 PFU ml⁻¹ phages to cells with different lengths was analogous even though the number of cells decreased considerably from 100 to 200 rpm (Figure 4.4a). This shows that two parameters highly influenced the amount of phages produced inside a cell. First, phage progeny production appeared to be closely related to the cell length as the more lengthened a cell was the more progeny phages were produced (Figure 4.4a), and

secondly, like observed in the temperature experiments, the specific growth rate seems to be directly responsible for the number of new phages (Table 4.2).

Table 4.2 – Influence of rotary agitation speed on cell size, growth rate and influence of different phage concentrations on phage infection parameters and cell density decrease (%).

Speed (rpm)	Cell size* (μm)	μ (h^{-1}) (\pm SD)	Log (CFU ml^{-1})	Log (PFU ml^{-1})	Beginning of infection (min)	Lysis period (min)	Density decrease (%) (\pm SD)
0	1.5–3.3	0.048 (0.004)	9.88 (0.04)	9	40	260	27.04 (4.50)
				8	40	260	33.19 (4.22)
				7	80	220	31.39 (3.09)
100	2.7–13.0	0.050 (0.001)		9	80	100	81.98 (3.62)
				8	80	140	80.16 (2.70)
				7	160	140	79.30 (3.27)
160	4.2–23.8	0.084 (0.003)	8.77 (0.12)	9	40	120	79.75 (6.75)
				8	60	120	78.11 (7.96)
				7	100	80	69.67 (7.62)
200	11.3–29.2	0.127 (0.002)	7.58 (0.04)	9	60	80	78.73 (3.79)
				8	80	120	85.25 (1.81)
				7	100	120	84.15 (2.38)

* minimum and maximum size from measurements of 5-10 cells, SD means standard deviations

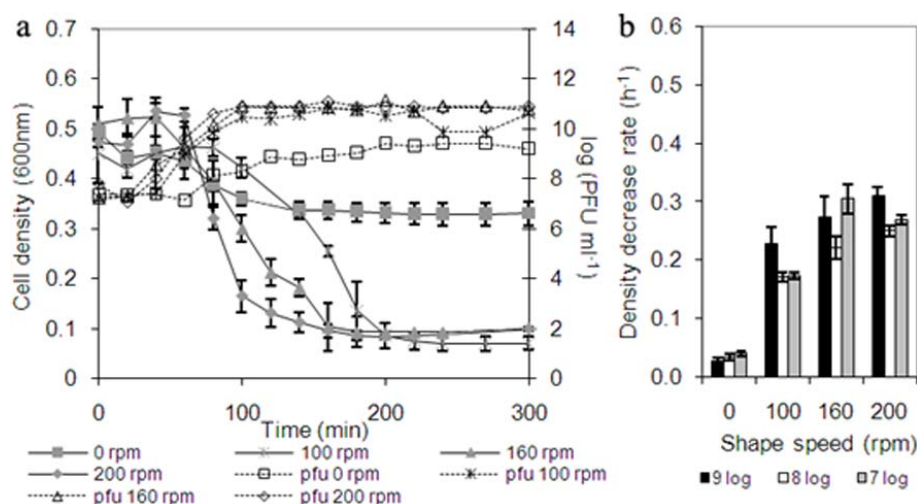


Figure 4.4 – Infection of *P. fluorescens* cells grown and infected with phage ϕ IBB-PF7A under varied shake speeds. Measurement of a) cell density and phage numbers at different time periods using a phage concentration of 1×10^9 PFU ml^{-1} and 30°C and b) cell decrease rates obtained after infection of cells with different phage concentrations. Error bars indicate standard deviations of 24 different wells.

This study shows that *P. fluorescens* morphology and length were greatly influenced by the rotary agitation applied during the growth of the cultures. Furthermore, the phage infection process was dependent of the application of agitation, as cell lysis was reduced when no speed was applied. Moreover, two factors appear to be directly influencing the production of new phages: cell length and specific growth rate.

Effect of growth phase

In nature cells reside predominantly in biofilm communities where cells exist in different growth phases. This work investigates the effect of filamentous cells at different growth phases in the phage lytic efficacy. For the formation of filamentous type of cells, two parameters were chosen – agitation speed at 160 rpm and temperature at 30 °C. Cells were grown under these two constant conditions, until reaching four different growth phases: early-, mid- and late-logarithmic and stationary. Afterwards, the cultures were challenged with phages.

Cells at early-, mid-, and late-logarithmic phases were well infected by the different phage concentrations, showing similar density decrease patterns (Figure 4.5a). Contrarily to cells at the logarithmic phases, once the cells reached the stationary phase the phage infection process suffered a considerable change. Stationary cells challenged with phages were not readily lysed. First there was observed a quite long period where the density increased and only after this density increase period the cells started lysing. Interestingly, once the lysis had begun the process was surprisingly fast (Figure 4.5a). Phage progeny increase was fastest in mid-logarithmic cells and the concentration reached a plateau after 100 min of infection. In late-logarithmic cells, phage numbers reached this plateau 50 minutes later and in stationary cell infection this value is only reached after 120 min compared to mid-logarithmic cell infection (Figure 4.5a).

In terms of density decrease rate, phages were able to attack slightly faster cells at the mid-logarithmic phase (Figure 4.5b). The density decrease percentages obtained after phage challenge were slightly lower in late-logarithmic and stationary cells (Table 4.3). Nevertheless, the 70 % density reduction observed in these two phases was noticeably high and shows that the phage is in fact able to infect well cells at late growth phases and therefore their ability in controlling even 168 h biofilms.

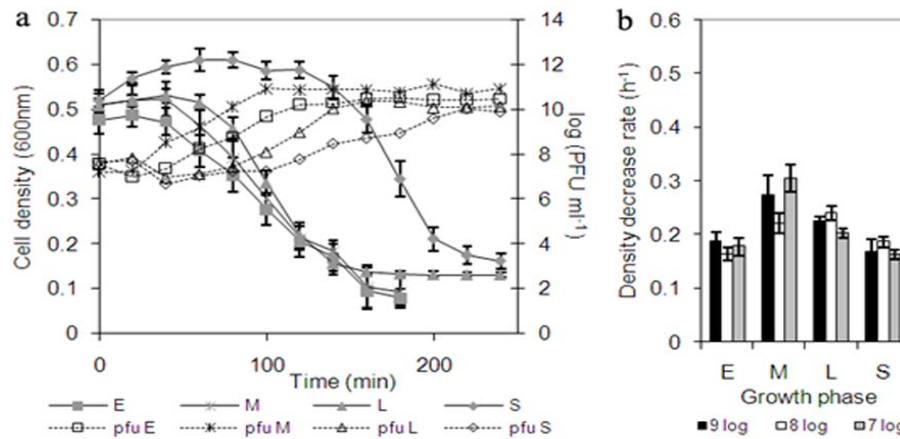


Figure 4.5 – Phage ϕ IBB-PF7A infection of *P. fluorescens* cells grown at different growth phases (early-log (E); mid-log (M); late-log (L) and stationary (S) phases). Measurement of a) cell density and phage numbers at different time periods using a phage concentration of 1×10^9 PFU ml⁻¹, 30 °C and 160 rpm; and b) cell decrease rates obtained after infection of cells with different phage concentrations. Error bars indicate standard deviations of 24 different wells.

Table 4.3 – Influence of *P. fluorescens* cell growth phase on cell size and influence of different phage concentrations on phage infection parameters and cell density decrease (%).

Growth phase	Log (PFU ml ⁻¹)	Beginning of infection (min)	Lysis period (min)	Density decrease (%) (\pm SD)
Early-log	9	40	120	79.80 (7.05)
	8	40	140	71.77 (4.63)
	7	60	120	64.97 (6.82)
Mid-log	9	40	120	79.75 (6.75)
	8	60	120	78.11 (7.96)
	7	100	80	69.67 (7.62)
Late-log	9	40	100	70.42 (2.93)
	8	80	100	70.74 (2.87)
	7	80	120	68.40 (2.52)
Stationary	9	80	140	71.42 (3.00)
	8	80	140	71.22 (2.43)
	7	80	140	57.36 (5.53)

SD means standard deviations

This work shows that filamentous cells under all logarithmic phases were similarly infected by phages. Moreover, the passage from log to stationary growth phase caused a

delay in the beginning of lysis. Nevertheless, significantly high density reductions were achieved even in stationary phase cells.

Discussion

Phages are ubiquitous in nature and have been studied with a variety of hosts. Different growth conditions were responsible for morphological changes in the *Pseudomonas fluorescens* cells, which either existed as rod or filamentous shaped. The phage used to infect the cultures, phage ϕ IBB-PF7A, was efficient in lysing both morphological cell types and two key factors were found to influence phage progeny production: cell length and growth rate. Also, phage ϕ IBB-PF7A showed efficacy towards stationary filamentous cells and infection was dependent on the phage-host encounter as lysis was extremely reduced under static conditions.

Cell morphology and length were found to be greatly influenced by rotary agitation and temperature. The typical rod-shape morphology was observed under stationary conditions (0 rpm) and when growth took place at temperatures below or equal to 25°C. Cells acquired filamentous morphology already at 100 rpm and higher agitation speeds as well as at 30°C. However, cells grown at 30°C and under non-agitation conditions exist as rod-shaped. So, for this *P. fluorescens* strain to become filamentous it was required a combination of high temperature and existence of agitation. The filamentous morphology was most likely due to a blocking of the cell division process as described for example in *Pseudomonas aeruginosa* and *Escherichia coli* (Greenwood & O'Grady 1973; Maki *et al.* 2000; Rolinson 1980; Steinberger *et al.* 2002; Werner *et al.* 2004; Wright *et al.* 1988; Yokochi *et al.* 2000). It is known that cell division requires several proteins and, in case one or more of these proteins become nonfunctional, the cells form non septated cells or filaments, that can have 50 times longer lengths. The only obvious effects are morphological growth rate, DNA replication, and chromosome segregation which all appear to continue as if nothing had happened. This cell division blocking doesn't stop cell growth and these cells still maintain motility (Errington *et al.* 2003; Margolin 2000; Rothfield *et al.* 1999).

Phage infection occurs when there is an interaction of phage proteins with molecules or structures, such as lipopolysaccharides or proteins and flagella or capsules that exist on the bacterial surface (Puig *et al.* 2001). So, although *P. fluorescens* cell morphology was

shown to alter, both filamentous and rod shaped cells were efficiently infected by phages. Therefore, it can be hypothesized that *P. fluorescens* filamentous shaped cells possessed the necessary receptors for phages to adsorb and start the reproduction cycle; otherwise the infection would have not taken place.

Phage reproduction is known to be affected by many factors (De Paepe & Taddei 2006; Moebus 1996; Sillankorva *et al.* 2004). Here, it was observed a direct relation of phage progeny with two parameters. First, temperature and rotary agitation influenced greatly the specific growth rate, with high rates resulting in more phages released from the infected cells. Second, lengthened cells were able to reproduce higher phage progeny numbers than rod shaped.

It has been frequently described, with different phage-host systems, that there is a reduced lysis when cells are near the stationary phase (Abedon & Yin 2006; Burch & Chao 2004; Haywood 1974; Middleboe 2000; Ricciuti 1972; Sillankorva *et al.* 2004). For instance, T4 phage cannot even produce a burst if the *E. coli* cells are in the stationary phase (Delbruck 1940; Hedon 1951). The main factors accounted for the reduction of lysis in stationary cells are: less phage-adsorption sites, lower phage progeny per infection, reduced cell lysis due to cell wall thickness or increased non-viable infections (Weitz & Dushoff 2007). However, in this work, stationary *P. fluorescens* cells and all the factors responsible for lysis reduction appear not to influence the activity of this specific phage. The main difference to logarithmic cell infection was that in stationary cells the start of phage infection was delayed due to an initial regrowth phase. However, after this initial cell growth phase, phage ϕ IBB-PF7A was able to lyse the newly produced cells as well as the stationary cells. Interestingly, the lysis period observed in stationary cells was exceptionally fast and comparable to the lysis period of early-, mid- and late-logarithmic cells and resulted in significant density reduction percentages. This confirms previous hypothesis, where it was observed that phage ϕ IBB-PF7A could reduce the number of cells, including stationary cells, from 24, 72, 120 and 168 h *P. fluorescens* biofilms (Sillankorva *et al.* 2008). This shows that the decrease of phage effectiveness so many times described once cells reach stationary or near stationary phases, is not universal and there are most certainly more phages, like phage ϕ IBB-PF7A, which are highly effective in infecting older cells.

It is known that the number of phages per number of host bacterium is determinant for the rapidity of phage infection. Contrarily to high phage ϕ IBB-PF7A concentrations, the

use of low concentrations caused a considerable delay on the beginning of cell lysis that was more pronounced under the influence of low temperature. Nevertheless, at low temperatures, these less concentrated phage solutions were interestingly capable to eliminate a higher number of cells.

Previously, it was shown that after a slight delay of infection, biofilms of *P. fluorescens* were well infected by phage ϕ IBB-PF7A under static (non-agitation) conditions. This was mostly due to a high number of progeny phages that remained in the proximity of the infected neighboring biofilm cells (Sillankorva *et al.* 2008). Contrarily to these results, planktonic cells were inefficiently infected by phage under non-agitated conditions (0 rpm). It appears that phages are not encountering their hosts and due to this fact the infection process is extremely reduced.

In conclusion, this is a novel study where a newly isolated phage ϕ IBB-PF7A was shown to infect filamentous and rod-shaped cells. Furthermore, this phage was highly efficient in attacking stationary cells and this is a clearly an important fact which makes possible phage application to real environments where cells are definitely present in different growth stages.

Materials and Methods

Bacteria and bacteriophage

Pseudomonas fluorescens PF7 was isolated from a dairy plant. The bacterium was grown at 30 °C in Tryptic Soy Broth (TSB, Fluka) or in solid TSA medium that contains 1.2 % (wt/vol) of Bacto™ agar (Difco). The bacterium was subcultured once and glycerol stocks were performed and stored frozen at –80 °C until further use. Bacteriophages ϕ IBB-PF7A was isolated from raw sewage and purified and concentrated using the double soft agar layer (Sambrook & Russell 2001) where the soft agar top-layer of TSB contained 0.6 % of Bacto™ agar.

Phage titration of stock solution

Bacteriophage of ϕ IBB-PF7A titers were analyzed as described by Adams (Adams 1959).

Effect of different parameters on cells

P. fluorescens were grown at different temperatures (5°C to 30 °C), rotational agitation speeds (0 rpm to 200 rpm) (Multitron, Infors AG, Bottmingen-Basel, Switzerland) and during different time periods to have cells in distinct growth phases (early exponential, mid exponential, late exponential and stationary). Cells were grown in Erlenmeyers with 100 ml of TSB. After growth, the cultures were centrifuged (9,000 rpm, 10 min, 4 °C) and the pellet was resuspended in used TSB (supernatant from the centrifugation performed). The optical density (600 nm) of cells, grown at 30°C using different shake speeds, was adjusted to 0.5 and colony forming units were performed.

Phage infection of *P. fluorescens* cells

After adjusting the OD₆₀₀ to 1.0 with used TSB, 125 μ l of *P. fluorescens* cultures, grown under different conditions, were put on the wells of a 96-well microplate. Phage ϕ IBB-PF7A solutions (125 μ l) with different concentrations (10^7 , 10^8 and 10^9 PFU ml⁻¹) were added to the wells. Control experiments were performed with 125 μ l of suspension and 125 μ l of SM buffer (5.8 g l⁻¹ NaCl, 2 g l⁻¹ MgSO₄.7H₂O, 50 ml l⁻¹ 1M TRIS, pH 7.5). The duration of phage infection was dependent on the rapidity of phages to decrease the cell density. OD measurements were performed at different time intervals to evaluate

phage lytic parameters (beginning of lysis and lysis period) and to determine the cell density decrease percentage and rate (h^{-1}).

Field Emission Scanning Electron Microscopy (FESEM)

Samples (100 μl) grown under different conditions (temperature and rotational agitation) were put on stainless steel slides and fixed with 500 μl of 2.5 % glutaraldehyde and incubated at 4 °C for 1 h. Afterwards, samples were washed by immersion on phosphate buffer saline (PBS) and dehydrated in ethanol series, followed by critical drying (Critical Point Dryer CPD 030). Samples were coated with platinum and analyzed with FESEM in a JEOL JSM-6300F (Tokyo, Japan) instrument.

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1. LITERATURE REVIEW
2. ISOLATION AND CHARACTERIZATION OF PHAGES
3. PHAGE BIOFILM INTERACTION
4. PHAGE AND PLANKTONIC CELLS

5. MAIN CONCLUSIONS AND SUGGESTIONS FOR FORTHCOMING WORK

5.1 Main conclusions

The goal of this thesis was to select suitable phages candidates and verify their capability of controlling single and dual species bacterial biofilms. Thus, the core of this thesis was divided into distinct chapters, each of which aiming at bringing more insights about different phage aspects.

The first experimental work described of this thesis consisted in phage isolation and, principally, selection and characterization of phages for their further use in control experiments. As different environments have their own precise niche of predominant and characteristic spoilage and pathogenic bacteria arises the need to isolate new phages. Collection phages, especially the phages with broad host range, are suitable to be applied in most circumstances; nevertheless, phages from a particular environment usually prove to be more efficient. Phage lytic profiles, performed against different bacterial isolates, are required to exclude temperate phages and are a good strategy to select the best or broadest host range phages. The study of phage growth cycle characteristics is essential for all future infection experiments, whether performed with planktonic or biofilm bacteria. Phage characterization is equally useful for their classification and genomic sequencing can elucidate about genes present and their function on the phage replication properties. Furthermore, genome sequencing can be significant in the future development of phage or phage-based products and also of utmost importance if genetic modifications of phages are desired. A thorough characterization of the two phages, ϕ IBB-PF7A for *P. fluorescens* and ϕ IBB-SL58B for *S. lentus*, was performed. Genome sequencing of ϕ IBB-PF7A allowed the formation of a new sub-group, within the T7-supergroup of phages. This new sub-group has two representative phages: ϕ IBB-PF7A for *P. fluorescens* and gh-1 of *P. putida*. These phages resemble with each other at protein level approximately 55 %, however ϕ IBB-PF7A possess more putative genes. Taking into account that phages are suggested to be the predominant lifeform in the biosphere, it can be affirmed that phage sampling and isolation is scarce. Thus, it is not unexpected to discover new phages. An example of that is phage ϕ IBB-SL58B for *S. lentus* which reveals no morphological, physico-chemical and even genetical resemblance with any phage described so far and is most likely a novel type of phage belonging to the *Podoviridae* family. This *Staphylococcus* phage is also the first polyvalent phage which does not belong to the *Myoviridae* family.

After characterization of phages, emphasis was given to biofilm-phage interaction studies. The single species biofilms studied here were quite well attacked by their specific phages and phages decreased a significant number of viable cells. However, factors involved in biofilm formation and applied during phage infection affected the phage killing efficacy. Moreover, the two phages assumed different biofilm infection behaviours. Phage ϕ IBB-PF7A infected best *P. fluorescens* biofilms formed and infected under non-shaken conditions and the best infection of *S. lentus* biofilm was unexpectedly observed after infection of biofilms formed under severe starvation conditions. In *P. fluorescens* biofilms, cell lysis due to phage ϕ IBB-PF7A infection started faster in younger biofilms, but already after 4 h of treatment the level of surviving cells was approximately the same number in 24 up to 168 hours old biofilms. Also, this phage was productive in infecting both rod and filamentous shaped biofilm and planktonic cells. Furthermore, the *P. fluorescens* phage proved also to be excellent in killing both planktonic and biofilm cells at the stationary phase as demonstrated in Chapters 3 and Chapter 4. The work described in this thesis proves that there are phages that can effectively reproduce in their hosts grown under severe starvation, assuming different morphologies (rod and filamentous) and even hosts at the stationary growth stage.

Phage infection was also investigated with dual species biofilms. Phage cocktail application resulted in significant cell reductions from the different dual species biofilms. The phage cocktail was also highly efficient in controlling planktonic bacteria released from the biofilms. The infection of dual species biofilms assumed similar patterns to those observed in experiments of single biofilm infections carried out with phage ϕ IBB-SL58B and *S. lentus*. This was somewhat expected as this bacterium is dominant in the referred biofilms. Phage treatment of mixed biofilms was influenced by the same parameters as the ones affecting infection of single species biofilms of *S. lentus*. Furthermore, biofilms formed and infected under non-shaken conditions revealed to be inefficiently reduced. This can be due to: i) protection of the hosts against their respective phages due to the presence of non-susceptible bacterium, ii) to an inefficient phage-host interaction due to a lack of convection mechanisms, iii) to a different content and amount of extracellular polysaccharides and proteins in shaken and non-shaken biofilms that can be preventing phages from reaching the specific receptors. However, protection of the hosts from phage attack due to the presence of a non-susceptible bacterium can possibly be ruled out, as this work demonstrated that a single phage, specific for the less

predominant bacterium, easily reached and efficiently killed the target host within the dual species biofilms. It was also verified that a single phage applied to a dual species biofilm destroyed partly the biofilms as, after phage treatment, a high amount of cells, suggestedly of the non-susceptible bacterium, were released from the dual species biofilms to the planktonic phase. This proves that phages can be adopted as a method to kill a specific bacterium even when its host resides in mixed consortium.

Although phages can decrease bacterial populations present in biofilms, these biological agents alone are most likely not efficient enough to be applied to control industrial biofilms. Commonly used cleaning procedures remove not only microorganisms but also all undesirable materials (e.g. foreign bodies, cleaning chemicals, soil, etc.) and phages are not capable of this task. However, phages could have a similar function to nowadays used biocides and sanitizers, and be used after the major cleaning processes, to kill specific bacterium on the remainder biofilms. Aqueous phage solutions could be applied to rinse surfaces through spraying, painting, coating, pouring or immersion of inanimate objects. Also, phages could be coupled with conventional cleaners - detergents, disinfectants, ammonium-based chemicals, however phage stability and biological activity needs to be reassured. This strategy could reduce the chemical loads applied during disinfection procedures adopted in industrial facilities.

5.2 Suggestions for forthcoming work

Several studies can be suggested for future work. For instance, single phages or phage cocktails could be coupled with antimicrobial agents and tested on biofilms formed by far more than two species or could even be tested in real industrial biofilms.

Also, now that the phage sequences are known (or partly known) another future study could involve their genetic manipulation to “build” less host specific phages. This could be accomplished by changing the genes responsible for host specificity, such as the proteins in the tail fibers. Gene manipulation could also be used to include a polysaccharide lyase to improve the polymeric matrix degradation.

Furthermore, phage receptors (ex: outer membrane proteins and lipopolysaccharides) present on rod and filamentous shaped *P. fluorescens* should be analysed. Also, the phage growth cycles in the distinct length cells could be performed to study the amount of cells reproduced inside these hosts.