MONIQUE RENON ELLER

# ISOLATION AND CHARACTERIZATION OF A *PSEUDOMONAS*-SPECIFIC PHAGE AND ITS USE TO CONTROL MILK PROTEOLYSIS

Dissertation thesis presented to the Universidade Federal de Viçosa as part of the requirements of the PostGraduate Program in Agricultural Microbiology, to obtain the title of *Doctor Scientiae*.

VIÇOSA
MINAS GERAIS - BRASIL
2012

**Ficha catalográfica preparada pela Seção de Catalogação e Classificação da Biblioteca Central da UFV**

T
E45i
2012

Eller, Monique Renon, 1986-
Isolation and characterization of a *Pseudomonas*-specific phage and its use to control milk proteolysis / Monique Renon Eller. – Viçosa, MG, 2012.
xiv, 115f. : il. ; (algumas color.) ; 29cm.

Texto em português e inglês
Orientador: Sérgio Oliveira de Paula
Tese (doutorado) - Universidade Federal de Viçosa.
Inclui bibliografia.

1. Bacteriófagos. 2. *Pseudomonas*. 3. Genoma. 4. Leite - Qualidade. 5. Leite - Contaminação. 6. Leite - Microbiologia. I. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-Graduação em Microbiologia Agrícola. II. Título.

CDD 22. ed. 579.26

MONIQUE RENON ELLER

# ISOLATION AND CHARACTERIZATION OF A *PSEUDOMONAS*-SPECIFIC PHAGE AND ITS USE TO CONTROL MILK PROTEOLYSIS

Dissertation thesis presented to the Universidade Federal de Viçosa as part of the requirements of the PostGraduate Program in Agricultural Microbiology, to obtain the title of *Doctor Scientiae*.

APROVED: December, 06th, 2012

| | |
|---|---|
| Prof. Antônio Fernandes de Carvalho (Co-advisor) | Prof.ª Daniele da Glória de Souza |
| Prof.ª Márcia Rogéria de Almeida | Prof. Luciano Gomes Fietto |

Prof. Sérgio Oliveira de Paula
(Advisor)

*To my parents, sisters and brother, and to my beloved husband. My safe harbor.*

“They say I'm a lucky man ... I just know that the more I work, the luckier I am” (Anthony Robbins).

## Acknowledgments

To God, for my life, health, friends, family, and for the providence.

To Universidade Federal de Viçosa and to my masters and colleagues of the Department of Microbiology, for the opportunity, structure, and material and technical support. Special thanks to Nilcéia, for the patience and help.

To CAPES, for the finantial support to this research.

To my advisor, professor Sérgio Oliveira de Paula, for advice and encouragement, the good ideas and availability. To my co-advisors for their dedication, advice and availability.

To the colleagues of the Laboratory of Molecular Immunovirology, the the technicians, João and Jorge, for the friendship, teachings and availability. Special thanks to Roberto and Priscilla, for the daily work and learning. To Pedro, for the encouragement and technical aid.

To my friends:

Zamira, for the eternal friendship.

Raphael, Chris and Pituca, for the laughtes, tranquility and cheer on harder days.

Maura, for the friendship and company in the interminable hours of presentations and laboratory.

To Rafael, my dedicated husband, for the fellowship, patience, laughters, professional assistance and for make unforgettable moments from my routine. To his mother, Maria, for her dedication, teachings, faith and love of each day.

To all my family, for the good advices, talkings and concepts of life. In particular, to my parents, Romeu and Valéria, for the faith and unconditional strength; to my sisters, Pâmela and Allana, for the friendship and complicit; and to my brother Luan, for the lessons of life and love.

Anyway, to all who stood by my side and who directly or indirectly contributed to this realization, my thanks!

## List of Figures

# List of Tables

# Table of Contents

# Resumo

ELLER, Monique Renon. D.Sc. Universidade Federal de Viçosa, dezembro de 2012. **Isolamento e caracterização de um fago de *Pseudomonas* e seu uso no controle da proteólise do leite.** Orientador: Sérgio Oliveira de Paula; Co-orientadores: Antônio Fernandes de Carvalho, Cynthia Canedo da Silva, Leandro Licursi de Oliveira e Maria Cristina Dantas Vanetti.

O desenvolvimento de bactérias psicrotróficas no leite cru e a proteólise causada por suas enzimas termorresistentes podem causar efeitos graves sobre a qualidade dos produtos lácteos. Estudos envolvendo o controle de deterioração dos alimentos utilizando agentes biológicos têm aumentado na última década uma vez que o biocontrole geralmente não gera efeitos indesejáveis sobre a saúde humana e as características dos alimentos. Neste estudo, um bacteriófago de *Pseudomonas*, denominado UFV-P2, foi isolado a partir de águas residuais de um lacticínio. O fago foi capaz de reduzir a proteólise das α, β e κ-caseínas por uma estirpe de *Pseudomonas fluorescens* proteolítica, mas não reduziu a população de bactérias nesse meio. O fago UFV-P2 tem um genoma de DNA linear de 45.517 pb, sem genes de tRNA, um teor de GC de 51,5%, e 41 ORFs. As ORFs foram anotadas em cinco grupos de proteínas diferentes, um deles contendo 14 proteínas hipotéticas com função desconhecida (34,1%). Os grupos restantes consistiram de uma chaperona, quatro proteínas constitutivas e sete genes de proteínas estruturais, incluindo uma *major head*, uma proteína portal, e uma proteína da cauda. Finalmente, 15 ORFs (36,6%) foram anotadas com genes que codificam enzimas, incluindo uma lisozima, as subunidades da terminase, uma endonuclease, as duas partes da DNA Polimerase e uma primase / helicase. A sequência completa do genoma foi depositada no GenBank sob o número de acesso JX863101. Análises comparativas genômicas e estruturais mostraram que o fago UFV-P2 tem uma organização semelhante a dos genomas dos fagos

MR299-2, PaP3 e LUZ24, recentemente agrupados no grupo LUZ24-like, o que nos levou a propor a classificação do UFV-P2 nesse mesmo gênero. Além disso, nós propusemos a inclusão do fago tf, anteriormente não classificado, neste gênero. Um estudo foi realizado a fim de avaliar a potencial viabilidade econômica de um produto gerado a partir do fago isolado. Ele mostrou que o uso desta tecnologia possui várias vantagens, e que a sua consolidação e integração dependem dos esforços bem definidos em pesquisa e desenvolvimento.

## Abstract

ELLER, Monique Renon. D.Sc. Universidade Federal de Viçosa, December, 2012. **Isolation and characterization of a *Pseudomonas*-specific phage and its use to control milk proteolysis**. Advisor: Sérgio Oliveira de Paula; Co-advisors: Antônio Fernandes de Carvalho, Cynthia Canedo da Silva, Leandro Licursi de Oliveira and Maria Cristina Dantas Vanetti.

The development of psychrotrophic bacteria infecting raw milk and the proteolysis caused by their proteolytic heat-resistant enzymes can cause serious effects on the quality of dairy products. Studies involving the control of food spoilage using biological agents have emerged in the last decade because biocontrol generally has no consequences on human health and food characteristics. In this study, we isolated a *Pseudomonas*-phage, named UFV-P2, from waste water of a dairy factory. The phage was able to reduce the proteolysis of α, β e κ-caseins by a proteolytic strain of *Pseudomonas fluorescens*, but did not reduce bacterial population in this environment. UFV-P2 has a linear 45,517 bp DNA genome with no tRNA genes, a GC content of 51.5%, and 41 ORFs. The ORFs analyzed were annotated to five different protein groups, one of them containing 14 hypothetical proteins with unknown function (34.1%). The remaining groups consisted of one chaperone, four constitutive, and seven structural protein genes, including a major head, a portal, and a tail fiber protein. Finally, 15 ORFs (36.6%) hits with genes coding enzymes, including one lysozyme, the terminase subunits, an endonuclease, the two parts of the DNA polymerase and a primase/helicase. The complete genome sequence was deposited in GenBank under accession number JX863101. Genomic and structural comparative analyses showed that UFV-P2 has a genome organization similar to the MR299-2, PaP3 and LUZ24 phage genomes, recently grouped as LUZ24-like viruses, what lead us to propose the classification of UFV-P2 in the LUZ24-like genus. Additionally,

we propose the inclusion of the previously unclassified phage tf in this genus. Studies carried out were performed to evaluate the potential economic viability of a product generated from the isolated phage. It showed that the use of this technology has a number of advantages, and that the consolidation and integration of technology depends on the well-defined efforts in research and development.

# 1. GENERAL INTRODUCTION

Milk proteolysis, when uncontrolled or unwanted, cause serious flavor and taste defects in the product, which cannot be passed to the consumer. The problem is even greater when it is caused by enzymes of psychrotrophic bacteria that maintain their activity even after heat treatments, including UHT. These enzymes slowly hydrolyze the milk caseins, increasing the milk viscosity and causing the effect called gelation of UHT milk, very difficult to be discovered by the manufacturers before its transfer to the final consumer.

Thus, preventive measures consist of the only way to eliminate this problem in the dairy industry, and the use of phages could be an economical, practical and safe alternative. Bacteriophages are viruses capable of killing the host bacterium or partially inhibit its metabolism. The addition of these particles in the food or even as cleaning agents doesn't cause changes in the characteristics of the food nor risks to human health.

The main genus of psychrotrophic bacteria found in milk is *Pseudomonas*. The studies about specific phages against these bacteria are limited mainly to the use of phages to control *P. aeruginosa* in hospitals, and there are few isolated and characterized phages for the control of *P. fluorescens*, the main species found in the dairy environment. Thus, we propose the isolation and characterization of a phage that infects the bacteria *P. fluorescens* and the analysis of its potential use as a food preservative or as a component of active sanitizers in food industries.

This work consists of a series of articles describing the main aspects of proteolysis in milk contaminated with psychrotrophic bacteria (Chapter 2), the complete genome analysis of the phage UFV-P2, isolated from waste water from a dairy industry (Chapter 3), and the capacity of this phage to

inhibit milk proteolysis by *Pseudomonas fluorescens* 07A (Chapter 4). Finally, Chapter 5 presents a complete study of economic viability of the technology of using phages to control milk proteolysis.

# 2. LITERATURE REVIEW

# Use of phages to control milk proteolysis by termostable enzymes of psychrotrophic bacteria

*Abstract*

Studies involving the control of food spoilage using biological agents have emerged in the last decade because biocontrol generally has no consequences on human health and food characteristics. The new interest in the use of phages to control pathogenic bacteria expanded to industrial microbiology and is shown to be a safe, economic, and efficient technology. This study reviews the main aspects of the use of phages to control food contaminants, with an emphasis on the control of milk proteolysis by proteases from psychrotrophic bacteria, the main cause of UHT milk gelation.

Key words: Bacteriophage; Milk proteolysis; Biocontrol; Food microbiology; Food spoilage; *Pseudomonas*.

## *Use of phages to control food contaminants*

Bacteriophages are viruses responsible for the elimination of 4 to 50% of the bacterial cells produced every day, significantly contributing to biogeochemical cycles and acting as a reservoir for genetic diversity on earth (Suttle, 2005). They can be used as a natural, highly specific, non-toxic, and accessible tool to control microorganisms. Thus, much has been studied about their use and the use of their enzymes in treating clinical infections of humans and animals, or phagotherapy (Fischetti, 2010; Gorski et al., 2009; Housby

and Mann, 2009). Moreover, interest has increased in the use of these viruses for the biocontrol of bacterial contaminants in industrial environments. In particular, this increased interest is reflected in studies on the use of these agents in the food industry to control potentially pathogenic bacteria such as *Listeria monocytogenes* and *Salmonella* sp. (Jones et al., 1991; Loessner and Carlton, 2005). More recently, phages were found to inhibit growth of spoilage bacteria, which can cause significant economic losses every year (Arcuri et al., 2008; Azeredo and Sutherland, 2008; Sillankorva et al., 2008). Despite this information, studies on bacteriophages in the food industry are often related to the presence of phages that infect starter cultures, causing fermentation processes to fail (Eller et al., 2012). Research on the isolation of *Pseudomonas* phages still concentrates on the control of hospital contamination by bacteria *P. aeruginosa*, but there are few studies on the isolation of phages to control *P. fluorescens* (Sillankorva et al., 2008), an important agent of milk proteolysis. To date, only 78 *Pseudomonas* phage genome sequences exist in the EMBL-EBI database (http://www.ebi.ac.uk/genomes), a low number considering the importance of this contaminant and its estimated viral diversity. Of these, only three sequences correspond to *P fluorescens* phages, the UFV-P2 (Eller et al., 2013), phi-2 (Paterson et al., 2010) and phiIBB-PF7A (Sillankorva et al., 2011) phages.

### *Proteolysis in the dairy industry*

Proteolysis is the most complex biochemical event that occurs in most cheeses during cheese ripening and has been widely studied given its importance in the production of dairy products. The amino acids released

during the maturation process are used as substrates for a variety of catabolic reactions that generate compounds important to the development of the characteristic flavor of each kind of cheese and that depend on the dairy native microbiota (for example, non-starter lactic acid bacteria) or microorganisms added to the curd before ripening (Yvon and Rijnen, 2001). However, although proteolysis has beneficial effects and is essential for the development of desirable qualities of food products, uncontrolled proteolysis can negatively affect food quality.

The development of psychrotrophic bacteria infecting raw milk and the proteolysis caused by their proteolytic heat-resistant enzymes can cause serious effects on the quality of dairy products, such as off-flavors in pasteurized milk, hard cheeses, cottage cheeses, butter, and yogurt; reduced shelf life in pasteurized and Ultra-High-Temperature (UHT) milk; foaming formation in pasteurized milk and reconstituted milk powder; reduced thermal stability in milk powder and UHT milk; and reduced yields in cheese production (Sorhaug and Stepaniak, 1997).

The proteolysis of UHT milk by these enzymes during storage at room temperature is the main factor that limits its shelf life, resulting from changes in its flavor and texture (Datta and Deeth, 2003) and characterized by an increase in viscosity that, in some cases, leads to gel formation and a grayish, slightly rancid and filthy and bitter off-flavor (Datta and Deeth, 2001; Jonghe et al., 2001; Law et al., 1977; Meer et al., 1991; Sorhaug and Stepaniak, 1997). Thus, the shelf life of UHT milk depends on the number of psychrotrophic microorganism contaminants of raw milk, ranging from two to longer than 20 weeks.

Independent studies performed by Deeth et al. (2002) and López-Fandiño et al. (1993) showed significant differences in the behavior of UHT skimmed and whole milk contaminated with psychrotrophic microorganisms. Although skimmed milk exhibited a predominantly bitter flavor, whole milk presented a sour and acid flavor. The studies indicated that the different flavors occurred from increased proteolysis in skim milk caused by larger production of protease and susceptibility of proteins to attack by this enzyme. Furthermore, whole milk also involves the process of lipolysis caused by substantial amounts of lipase produced by contaminants (Deeth et al., 2002). This different behavior should be considered when establishing processing conditions.

A study performed in Brazil indicated that 30.3% of milk presented gelation to a certain degree (Tamanini et al., 2011). Dairy product proteolysis is caused by the natural milk proteases and/or thermostable proteases produced by psychrotrophic microorganism contaminants of raw milk during refrigeration. These proteases differ in their specificity for milk proteins (Nielsen, 2002). Datta and Deeth (2003) observed differences in milk proteolysis caused by plasmin and by a protease from *Pseudomonas*. The latter led to the formation of a hard gel, concluding that the *Pseudomonas* protease preferentially hydrolyzes the hydrophilic glycomacropeptide of κ-casein on the outside of the casein micelle. This hydrolyzation keeps the casein micelles intact and reduces steric repulsion between them, allowing the formation of a more compact gel. However, milk proteolysis by plasmin caused partial digestion of caseins through preferential hydrolysis of β-casein located inside the micelle, thus destroying it and inhibiting the formation of a stronger gel.

The undesirable changes occurring in the UHT milk produced from contaminated raw milk are also caused, or at least accelerated, by the casein hydrolysis and subsequent release of the β-lactoglobulin-κ-casein complex (βκ complex) formed during heat treatment. The complex forms a three-dimensional network of interconnected proteins, which causes gel formation (MacMahon, 1996). Thus, any processing (e.g. UHT treatment) or storage (e.g. refrigeration) condition that accelerates (or delay) the release of βκ complex from the casein micelle will accelerate (or decelerate) the time for gelation of UHT milk (Datta and Deeth, 2003).

Several studies reported the biochemical properties of proteases produced by some strains of *P. fluorescens* associated with dairy product spoilage (Dufour et al., 2008; Marchand et al., 2009). In milk, proteases of psychrotrophic bacteria preferentially hydrolyze κ-casein, β-casein, and then αS1-casein. Whey proteins are only weakly hydrolyzed (Chen et al., 2003; Dufour et al., 2008; Koka and Weimer, 2000; Rajmohan et al., 2002).

Baruzzi and colleagues (2012) analyzed the proteolytic activity of proteases of *Pseudomonas* in mozzarella cheese. They observed a higher proteolytic activity on the outside, which was in direct contact with a liquid contaminated with the proteolytic samples, originally used in the conservation of cheese. According to them, this phenomenon is consistent with the wrinkling and exfoliation that occur on the surface of mozzarella cheese, the progressive release of cheese cuts, and an increase in the turbidity of the liquid.

In addition to this problem, in the 1970s researchers Adams, Barach, and Speck (1975) already reported an increase in the coagulation of casein

attributable to protease activity of psychrotrophics after heat treatment. This phenomenon in coagulation is particularly important for UHT milk because it is intensified when heating at higher temperatures.

The genus *Pseudomonas* represents a number of bacterial groups found in a wide variety of environments. Some are plants, animals, and humans pathogens, whereas others are responsible for food contamination (Palleroni, 1992). *Pseudomonas* spp. are the major contaminating microorganisms that limit the shelf life of the processed fluid milk during refrigeration (Alatossava and Alatossava, 2006).

Among the bacteria belonging to this genus, the species *P. fluorescens* is the main food spoilage, especially in products of dairy origin (Dogan and Boor, 2003). This species comprises a large and heterogeneous group, which has been subdivided into five biotypes based on phenotypic characteristics (Palleroni, 1992). Beyond *P. fluorescens*, other species are associated with milk deterioration, such as *P. putida*, *P. fragi*,*P. maltophila*, *P. putrefaciens*, and, less frequently, *P. aeruginosa* (Alatossava and Alatossava, 2006; Wiedmann et al., 2000).

### *Bacterial biofilms in food industry*

The biofilm is a structure composed of a consortium of microorganisms colonizing a surface with different species that are integrated and surrounded by a complex structure formed by extracellular polymeric substances (EPS) that they produce (Costerton et al., 1987; Kumar and Anand, 1998; Moretro and Langsrud, 2004). The molecules that form the matrix include cell surface

proteins, pili, DNA, RNA, lipids, and, especially, polysaccharides (Flemming and Wingender, 2010; Rendueles et al., 2012). In the dairy industry, these biofilms consist primarily of milk organic components, in particular proteins and calcium phosphate, constituting a rich source for biofilm formation in equipment (Marchand et al., 2012; Sillankorva et al., 2008; Sorhaug and Stepaniak, 1197). Commonly, bacteria in the biofilm are catalysts of chemical reactions that corrode steel equipment (Gibson et al., 1999; Gram et al., 2007; Oliver et al., 2005). The presence of biofilms may also clog pipes, reduce heat flux through the surfaces, and contaminate food, although several studies found a decrease in the adhesion of certain microorganisms in the presence of milk or milk proteins (Al-Makhlafi et al., 1995; Barnes et al., 1999; Kumar and Anand, 1998).

The species of the microorganisms in biofilms and the substances that compose the EPS are functions of several factors, such as plankton population, composition of the raw materials, processing conditions, types of sanitizing, and equipment material. For example, thermo-sensitive bacteria of the genus *Pseudomonas* and *Listeria* are commonly found on equipment used in the steps before pasteurization, such as tubes and silos, whereas thermophilic biofilms are formed in equipment subjected to heating (Marchand et al., 2012).

Certain potentially pathogenic bacteria are found in biofilms in dairy industries, such as *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Salmonella* spp., *Escherichia coli* O157: H7, Shiga toxin-producing *E. coli*, and *Campylobacter jejuni* (Bore and Langsrud, 2005; Oliver et al., 2005). The initial colonization of certain biofilm-forming species may facilitate colonization by secondary, physiologically compatible species through the production of EPS, or inhibit

the adhesion of others. For example, microorganisms from rinse water, particularly *Pseudomonas*, *Aeromonas*, and *Legionella* spp., are able to form biofilms and act as a substrate for the anchoring of others with less potential adhesion microorganisms (Marchand et al., 2012). Sasahara and Zottola (1993) observed that *Listeria monocytogenes* formed a biofilm more efficiently when associated with a primary colonizing organism, the bacteria *Pseudomonas fragi*, which was attributed to polysaccharide production by this microorganism (Sasahara and Zottola, 1993). Currently, *Pseudomonas fragi* and *Flavobacterium* spp. are known to act as primary surface colonizers, facilitating the adhesion of *L. monocytogenes* cells, whereas the primary adhesion by *Bacillus* spp. and *Staphylococcus* spp. reduces adherence and biofilm formation by that bacterium (Moretro and Langsrud, 2004).

*Control points*

The use of phages in the control of contaminants in foodstuffs industries is based on three main points: limiting the growth of microorganisms in the raw material, control of biofilm in the points of the industrial process, and rapid and cheap detection of contaminated surfaces.

*Using phage as additives in the raw material:* In this case, the objective is to prevent biofilm formation on equipment and surfaces during the storage and transportation of raw materials by reducing the growth of, or even eliminating, psychrotrophic and mesophilic bacterial cells. The addition of phage in the raw material could reduce problems of undesired proteolysis of dairy products by psychrotrophic bacteria such pseudomonads.

*Using phage as sanitizers*: The second point is the use of phages to decontaminate surfaces and eliminate biofilms on equipment and utensils. Given its high penetration ability, phages in suspension may be used in places that are hardly reached and that commonly have bacterial biofilms. Furthermore, phages are able to efficiently reduce the bacterial population in biofilms even without prolonged exposure times, as demonstrated by Sillankorva and colleagues using *Pseudomonas*-specific phages (Sillankorva et al., 2010). However, infection of cells in an EPS matrix depends on biofilm chemical composition and environmental factors such as temperature, stage of development, physico-chemical conditions of the medium, and phage concentration, which increase the need for exhaustive studies until the phages can be used in foodstuffs (Chaignon et al., 2007; Sillankorva et al., 2010). In this context, genetic engineering has been of a great assistance in optimizing biofilm inactivation techniques and even increasing viral efficiency for this purpose, as was done in the work of Lu and Collins (2007). They altered the viral genome by inserting a gene that encodes an enzyme capable of degrading the polysaccharide matrix of biofilms. The modified phage was able to reduce *E. coli* biofilms by approximately 99.997% (Lu and Collis, 2007).

*Using modifying phages for spoilage detection:* The use of specific fluorescence-labeled phages (Fluorescent Bacteriophage Assay – FBA) (Goodridge et al., 1999), phages containing the gene for luciferase (Luciferase Reporter Phage – LRP) (Ulitzur and Kuhn, 2000), or phages that encode the green fluorescent protein (GFP) (Funatsu et al., 2002) have been considered an economically viable, sensitive, practical, and fast alternative for the detection of microorganisms in a number of environments. For example, a *Salmonella*-specific phage was engineered to detect and even quantify this pathogen in eggs (Chen and Griffiths, 1996).A recombinant protein isolated from a

*Salmonella* phage is commercially used to detect this pathogen in foods (VIDAS™UP Salmonella – BioMérieux Industry, França) as a modified and optimized protocol of the traditional method VIDAS, and results are achieved within 24 h. Likewise, a specific *L. monocytogenes* phage containing the *lux* gene was used to detect this contaminant in meat and showed great sensitivity and practicality, even in the presence of large numbers of natural competitors (Loessner et al., 1996). Thus, phages specific to the major agents of food spoilage, such as *P. fluorescens*, could be used to detect these microorganisms, helping to control contamination and enabling rapid implementation of corrective actions where necessary.

In industries, phages could act in combination with other components to improve the efficiency of treatment, such as antibiotics, enzymes, and chemical and/or physical agents which do not reduce viral activity. For example, some patents suggest using phages as active ingredients in disinfectants formulations (Breeuwer et al., 2005; Kang et al., 2012; Sulakvelidze et al., 2004; Yoon et al., 2011). In this case, the use of phages would decrease the need for high concentrations of sanitizers, thus reducing the risk of toxic residues in products brought to consumers.

### *Limitations in the use of phages in the food industry*

Although promising results have already been obtained, significant additional analysis is required before phages can be effectively used in food. The use of new additives and sanitization in the food industry require careful studies because residues may be encountered in products passed to consumers.

The use of phages in the food industry, either as additives or as active agents of sanitizers, also has additional implications.

*Specificity:* Generally, phages have a narrow host range, which can easily be countered using mixtures (cocktails) of phages specific for different hosts and / or host receptors. Research on the use of viral consortia and the interaction between different phages, and phages with different molecules present in food and in biofilms, is also fundamental to finding the best way to use these particles for biocontrol of food contaminants. However, phage specificity is also a positive point for its use. In addition to ensuring consumer safety, its specific activity does not destroy indigenous bacteria in raw material—unlike other microbial control agents—which is often required in the production of fermented derivatives.

*Resistance to acceptance:* The addition of viral particles in foods may be a factor for rejection by consumers. However, marketing programs emphasizing the beneficial effects of the use of a biological agent over a chemical reagent to control food spoilage could turn this limitation in a strategic advantage.

*Product registration*: Viruses are capable of carrying bacterial genes and transferring them from one bacterium to another, serving as backers of resistance or pathogenicity genes. Genomic sequencing has enabled the discovery of several genes involved in the transference of genetic material and triggering pathogenicity by the host (Koonin and Wolf, 2012). Thus, selecting phages lacking these genes is possible to enable their use for biocontrol in the food industry.

*Complex environments*: One of the major problems that the application of phages in food must overcome is the diversity and complexity of the environments in which they will act. Viral activity is highly dependent on environmental conditions, and small changes could cause large loss in activity (Coffey et al., 2011). Therefore, research on viral activity over food contaminants must be carried out in the conditions in which the phages will be used, including composition, presence of food preservatives, pH, and temperature. The current demand for milder food preservation methods and the standardization of processes will assist in maintaining the activity of the viral particles.

*Emergence of resistant strains*: The constant possibility of the emergence of bacteria resistant to phages used for biocontrol is one of the main points regarding the viability of studies in this area. However, phages have a great ability to readapt and circumvent the bacterial defense mechanisms, one of the most significant advantages on their use to control contaminants. In most environments, a wide variety of phages and hosts is involved in continuous cycles of coevolution in which the emergence of phage-resistant hosts is important for the preservation of bacterial strains (Rohwer, 2003), whereas constant phage co-resistance threatens these new bacterial strains (Labrie et al., 2010).

As typically occurs in the formulation of commercial products, defining the strategies and the limitations and potentialities with respect to using this technique is necessary and must be considered. However, the phages would be used as a supplement to control contaminants and not as a substitute for traditional control agents. The concomitant use of a variety of control methods has been a trend in food technology ("technology of barriers"), primarily by

reducing the effects of each treatment on the physico-chemical and sensorial characteristics of the food, and on consumer health.

### *Importance in the study of phage genomics and proteomics*

The first report on the therapeutic use of a phage was a note written by Bruynoghe and Maisin (1921), who noticed a reduction in pain and swelling after injection of staphylococcal phages in a region of boils. However, the use of these organisms in the beginning of the last century was restricted to a decade of treatments indiscriminately carried out, and almost no significant research. This environment, coupled with the discovery of penicillin and search for new antibiotics, generated the false impression that phagotherapy was not a good alternative for controlling infections and virtually stopped research in this area by the end of the century. However, with the frequent appearance of antibiotic-resistant bacteria, in addition to societal pressure to search for environmentally safe alternatives, research intensified on the use of phages to control undesirable bacteria in the industrial sector. These studies usually aimed to determine the structural features of a virus, its infective capabilities, ability to inhibit proteolysis and biofilm formation, and host range. Recently, studies on viral genomics and proteomics joined this ceaseless quest for knowledge to enable the industrial application of these organisms. Everyday, new studies are published that communicate the isolation, sequencing, and characterization of new strains, suggesting their potential use in industry; however, no marketable product has been developed specifically for the dairy industry.

The study of phages plays a central role in some of the most significant discoveries in the biological sciences, from the identification of DNA as the genetic material to the development of recombinant technology. Phage-derived proteins or recombinant proteins produced in viral particles are often used as diagnostic and therapeutic agents and for drug discovery (Liu et al., 2004; Loeffler et al., 2001; Schuch et al., 2002; Smith et al., 2001). The continuous introduction of new sequencing technologies in the last decade has caused a dramatic increase in the number of completely sequenced phage genomes: from 40 in January 1997 to 1089 in October 2012 (Ceyssens, 2009). However, the annotation of a new sequenced genome generates approximately 50% of ORFs encoding proteins with unknown functions. Eller and colleagues (2013) conducted a study on the sequencing of a genome of a *Pseudomonas fluorescens* phage and found 92 predicted ORFs, of which only 41 (44.6%) showed significant similarity to ORFs already described. Of these 41, 15 (36.6%) were related to ORFs that encoded proteins of unknown function.

### *Current Status*

At least 15 patents exist that involve the use of phages to control contaminants, generally pathogenic bacteria, none of them specific related to control of *Pseudomonas* in milk. Records began from the 1980s and greatly increased during the last decade (Breeuwer et al., 2005; Donovan, 2011; Jones et al., 1991; Kang et al., 2012; Loessner and Carlton, 2005; Pasternack and Sulakvelidze, 2009; Sulakvelidze et al., 2004; Sulakvelidze et al., 2010; Yoon et al., 2011). The patents registered to date are based on two main points: the standardization of a method/solution to use phages to control biofilms and the registration of a specific phage and all of its potential applications. For

example, the patent of Breeuwer and colleagues (2005) deals with the use of *Cronobacter sakasakii*-specific phages in foods, sanitizers, and possible infections, and part of its specification refers to the use of these viruses to control this contaminant in dairy products. The patent registered by Sulakvelidze and colleagues (2004) is on a formulation containing phages for biocontrol of contaminants on surfaces and equipment, giving special attention to the methodology for the application of these agents. Despite these numerous patents, only one phage until today has been recognized as GRAS (Generally Recognized As Safe) by the U.S. Food and Drug Administration, and its application is limited to controlling *L. monocytogenes* in certain meat products (FDA, 2006).

Ceyssens and colleagues (2011) noted that small genomic variations "intra-species" have phenotypic consequences on essential applications of different phages. Distinct host ranges were observed between isolates with identical tail fibers and/or genomic regions of early genes, implying that smaller genomic alterations can cause a significant change in the spectrum of viral infectivity. Thus, the importance of genomic studies on the largest number of phages and a comparison of different sequences that combine the biological characteristics of the virus with their respective gene sequences can be realized. Then, expanding the knowledge of these organisms will be possible to ensure greater reliability and agility on the registration processes for use as food additives or as sanitizer agents for biological control in the dairy industry.

*Acknowledgments*

This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de

Aperfeiçoamento de Pessoal de Nível Superior (CAPES), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of this manuscript.

*References*

Adams, D.M., Barach, J.T., Speck, M.L., 1975. Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. Journal Series of the North Carolina Agricultural Experiment Station; Paper Number 4746.

Alatossava, M.P., Alatossava, T., 2006. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. Microbiol. Res. 161, 334-346.

Al-Makhlafi, H., Nasir, A., McGuire, J., Daeschel, M., 1995. Adhesion of *Listeria monocytogenes* to silica surfaces after sequential and competitive adsorption of bovine serum albumin and b-lactoglobulin. Appl. Environ. Microbiol. 61, 2013–2015.

Arcuri, E.F., da Silva, P.D.L., Brito, M.A.V.P., Brito, J.R.F., Lange, C.C., Magalhães, M.M.A., 2008. Counting, isolation and characterization of psychrotrophic bacteria from refrigerated raw milk. Ciência Rural 38, 2250-2255.

Azeredo, J., Sutherland, I. W., 2008. The use of phages for the removal of infectious biofilms. Curr. Pharm. Biotechnol. 9, 261-266.

Barnes, L.M., Lo, M.F., Adams, M.R., Chamberlain, A.H.L., 199. Effect of Milk Proteins on Adhesion of Bacteria to Stainless Steel Surfaces. Appl. Environ. Microbiol. 65, 4543-4548.

Baruzzi, F., Lagonigro, R., Quintieri, L., Morea, M., Caputo, L., 2012. Occurrence of non-lactic acid bacteria populations involved in protein hydrolysis of cold-stored high moisture Mozzarella cheese. Food Microbiol. 30, 37-44.

Bore, E., Langsrud, S., 2005. Characterization of micro-organisms isolated from dairy industry after cleaning and fogging disinfection with alkyl amine and peracetic acid. J. Appl. Microbiol. 98, 96–105.

Breeuwer, P., Boissin-Delaponte, C., Joosten, H., Lardeau, A., 2005. Isolated phages and their use as disinfectants in food or for sanitation of factory environments. [EP 1533369 A1] Ref Type: Patent.

Bruynoghe, R., Maisin, J., 1921. Essais de thérapeutique au moyen du bacteriophage du Staphylocoque. J. Compt. Rend. Soc. Biol. 85, 1120-1121.

Ceyssens, P.J., Glonti, T., Kropinski, N.M., Lavigne, R., Chanishvili, N., Kulakov, L., et al., 2011. Phenotypic and genotypic variations within a single bacteriophage species. Virol. J. 8, 134-138.

Ceyssens, P.J., 2009. Isolation and characterization of lytic bacteriophages infecting *Pseudomonas aeruginosa*. PhD Thesis from Katholieke Universiteit Leuven, Leuven, Belgium.

Chaignon, P., Sadovskaya, I., Ragunah, Ch., Ramasubbu, N., Kaplan, J.B., Jabbouri, S., 2007. Susceptibility of staphylococcal biofilms to

enzymatic treatments depends on their chemical composition. Appl. Microbiol. Biotechnol. 75, 125–132.

Chen, J., Griffiths, M.W., 1996. Luminescent *Salmonella* strains as real time reporters of growth and recovery from sublethal injury in food. Int. J. Food Microbiol. 31, 27-43.

Chen, L., Daniel, R.M., Coolbear, T., 2003. Detection and impact of protease and lipase activities in milk and milk powders. Int. Dairy J. 13, 255-275.

Coffey, B., Rivas, L., Duffy, G., Coffey, A., Ross, R.P., McAuliffe, O., 2011. Assessment of *Escherichia coli* O157:H7-specific bacteriophages e11/2 and e4/1c in model broth and hide environments. Int. J. Food Microbiol. 147, 188-194.

Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, T.J., 1987. Bacterial biofilms in nature and disease. Annu. Rev. Microbiol. 41, 435–464.

Datta, N., Deeth, H.C., 2001. Age gelation of UHT-milk - a review. Food Bioprod. Process 79, 197-210.

Datta, N., Deeth, H.C., 2003. Diagnosing the cause of proteolysis in UHT milk. Lebenson Wiss Technol. 36, 173-182.

Deeth, H.C., Khusniati, T., Datta, N., Wallace, R.B., 2002. Spoilage patterns of skim and whole milks. J. Dairy Res. 69, 227-241.

Dogan, B., Boor, K.J., 2003. Genetic diversity and spoilage potentials among *Pseudomonas* spp. isolated from fluid milk products and dairy processing plants. Appl. Environ. Microbiol. 69, 130–138.

Donovan, D.M., 2011. Specific lysis of staphylococcal pathogens by bacteriophage phi11 endolysin. [US 8,012,730] Ref Type: Patent.

Dufour, D., Nicodème, M., Perrin, C., Driou, A., Brusseaux, E., Humbert, G., et al., 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. Int. J. Food Microbiol. 125, 188-196.

Eller, M.R., Dias, R.S., Moraes, C.A., Carvalho, A.F., Oliveira, L.L., Silva, E.A.M., et al., 2012. Molecular characterization of a new lytic bacteriophage isolated from cheese whey. Arch. Virol., In press. PMID: 22865166.

Eller, M.R., Salgado, R.L., Vidigal, P.M.P., Alves, M.P., Dias, R.S., de Oliveira, L.L., et al., 2013. Complete genome sequence of the *Pseudomonas fluorescens* bacteriophage UFV-P2. Genome Annoucement. In press.

FDA: Food and Drug Administration. 2006. Food additives permitted for direct addition to food for human consumption; Bacteriophage preparation. Federal Register: Rules and Regulations, 71[160]. Ref Type: Report.

Fischetti, V.A., 2010. Bacteriophage endolysins: a novel anti-infective to control Gram-positive pathogens. Int. J. Med. Microbiol. 300, 357-362.

Flemming, H.C., Wingender, J., 2010. The biofilm matrix. Nature Rev. Microbiol. 8, 623–633.

Funatsu, T., Taniyama, T., Tajima, T., Tadakuma, H., Namiki, H., 2002. Rapid and sensitive detection method of a bacterium by using a GFP reporter phage. Microbiol. Immunol. 46, 365-369.

Gibson, H.J., Taylor, H., Hall, K.E., Holah, J.T., 1999. Effectiveness of cleaning techniques used in the food industry in terms of the removal of bacterial biofilms. J. Appl. Microbiol. 87, 41-48.

Goodridge, L., Chen, J., Griffiths, M., 1999. The use of a fluorescent bacteriophage assay for detection of *Escherichia coli* O157:H7 in inoculated ground beef and raw milk. Int. J. Food Microbiol. 47, 43-50.

Górski, A., Miedzybrodzki, R., Borysowski, J., Weber-Dabrowska, B., Lobocka, M., et al., 2009. Bacteriophage therapy for the treatment of infections. Curr. Opin. Investig. Drugs. 10, 766-774.

Gram. L., Bagge-Ravn, D., Ng, Y.Y., Gymoese, P., Vogel, B.F., 2007. Influence of food soiling matrix on cleaning and disinfection efficiency on surface attached *Listeria monocytogenes*. Food Control 18, 1165–1171.

Housby, J.N., Mann, N.H., 2009. Phage therapy. Drug Discov. Today 14, 536-540.

Jones, C. R., Rennie, G. K., & Moore, C. H. 1991. Use of viruses against undesirable microorganisms. [EP 0414304 A2] Ref Type: Patent.

Jonghe, V., Coorevits, A., Van Hoorde, K., Messens, W., Van Landschoot, A., De Vos, P., et al., 2001. Influence of storage conditions on the growth of *Pseudomonas* species in refrigerated raw milk. Appl. Environ. Microbiol. 77, 460-470.

Kang, I.H., Park, M.T., Cho, Y.W., Choi, H., Shin, S.A., 2012. Bacteriophage and antibacterial composition comprising the same. [US 8,148,131] Ref Type: Patent.

Koka, R., Weimer, B.C., 2000. Isolation and characterization of a protease from *Pseudomonas fluorescens* RO98. J. Appl. Microbiol. 89, 280-288.

Koonin, E.V., Wolf, Y.I., 2012. Evolution of microbes and viruses: a paradigm shift in evolutionary biology? Front. Cell. Infect. Microbiol. 119. Epub 2012 Sep 13. PMID: 22993722

Kumar, C.G., Anand, S.K., 1998. Significance of microbial biofilms in food industry: a review. Int. J. Food Microbiol. 42, 9–27.

Labrie, S.J., Samson, J.E., Moineau, S., 2010. Bacteriophage resistance mechanisms. Nature Rev. Microbiol. 8, 317-327.

Law, B.A., Andrews, A.T., Sharpe, M.E., 1977. Gelation of UHT sterilized milk by proteases from a strain of *Pseudomonas fluorescens*, isolated from raw milk. J. Dairy Res. 44, 145-148.

Liu, J., Dehbi, M., Moeck, G., Arhin, F., Bauda, P., Bergeron, D., et al., 2004. Antimicrobial drug discovery through bacteriophage genomics. Nature Biotechnol. 22, 185-191.

Loeffler, J.M., Nelson, D., Fischetti, V.A., 2001. Rapid killing of *Streptococcus pneumoniae* with a bacteriophage cell wall hydrolase. Science 294, 2170-2172.

Loessner, M., Carlton, R.M., 2005. Virulent phages to control *Listeria monocytogenes* in foodstuffs and in food processing plants. [US 2005/0175594 A1] Ref Type: Patent.

Loessner, M.J., Rees, C.E., Stewart, G.S., Scherer, S., 1996. Construction of luciferase reporter bacteriophage A511::luxAB for rapid and sensitive detection of viable *Listeria* cells. Appl. Environ. Microbiol. 62, 1133-1140.

López-Fandiño, R., Olano, A., Corzo, N., Ramos, M., 1993. Proteolysis during storage of UHT milk: differences between whole and skim milk. J. Dairy Res. 60, 339-347.

Lu, T.K., Collins, J.J., 2007. Dispersing biofilms with engineered enzymatic bacteriophage. P. Natl. Acad. Sci. USA 104, 11197-202.

Marchand, S., Block, J.D., Jonghe, V.D., Coorevits, A., Heyndrickx, M., Herman, L., 2012. Biofilm formation in milk production and processing environments; influence on milk quality and safety. Compr. Rev. Food Sci. F. 11, 133-147.

Marchand, S., Vandriesche, G., Coorevits, A., Coudijzer, K., De Jonghe, V., Dewettinck, K., et al., 2009. Heterogeneity of heat-resistant proteases from milk *Pseudomonas* species. Int. J. Food Microbiol. 133, 68-77.

McMahon, D.J., 1996. Age-gelation of UHT milk: Changes that occur during storage, their effect on shelf life and the mechanism by which age-gelation occurs. Heat treatments and alternative methods. International Dairy Federation ref S.I. 9602. Brussels, Belgium, International Dairy Federation, 315-325.

Meer, R.R., Bakker, J., Bodyfelt, F.W., Griffiths, M.W., 1991. Psychotrophic *Bacillus* spp. in fluid milk products: a review. J. Food Protect. 54, 969-979.

Møretrø, T., Langsrud, S., 2004. *Listeria monocytogenes*: biofilm formation and persistence in food processing environments. Biofilms 1, 107-121.

Nielsen, S.S., 2002. Plasmin System and Microbial Proteases in Milk: Characteristics, Roles, and Relationship. J. Agr. Food Chem. 50, 6628-6634.

Oliver, S.P., Jayarao, B.M., Almeida, R.A., 2005. Foodborne pathogens in milk and the dairy farm environment: food safety and public health implications. Foodborne Pathog. Dis. 2, 115-129.

Palleroni, N.J., 1992. Human- and animal-pathogenic pseudomonads. In: Balows, A., Truper, H.G., Dworkin, M., Harder, W., Schleifer, K.-H. (Eds.), The Prokaryotes. A Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Springer-Verlag, New-York, pp. 3086-3103.

Pasternack, G.R., Sulakvelidze, A., 2009. *Listeria monocytogenes* bacteriophage and uses thereof. [US 7,507,571] Ref Type: Patent.

Paterson, S., Vogwill, T., Buckling, A., Benmayor, R., Spiers, A.J., Thomson, N.R., et al., 2010. Antagonistic coevolution accelerates molecular evolution. Nature 464, 275-278.

Rajmohan, S., Dodd, C.E., Waites, W.M., 2002. Enzymes from isolates of *Pseudomonas fluorescens* involved in food spoilage. J. Appl. Microbiol. 93, 205-213.

Rendueles, O., Kaplan, J.B., Ghigo, J.M., 2012. Antibiofilm polysaccharides. Environ. Microbiol., In press. doi:10.1111/j.1462-2920.2012.02810.

Rohwer, F., 2003. Global phage diversity. Cell 113, 41.

Sasahara, K., Zottola, E.A., 1993. Biofilm formation by *Listeria monocytogenes* utilizes a primary colonizing microorganism in flowing systems. J. Food Protect. 56, 1022-1028.

Schuch, R., Nelson, D., Fischetti, V.A., 2002. A bacteriolytic agent that detects and kills *Bacillus anthracis*. Nature 418, 884-889.

Sillankorva, S., Kluskens, L.D., Lingohr, E.J., Kropinski, A.M., Neubauer, P., Azeredo, J., 2011. Complete genome sequence of the lytic *Pseudomonas fluorescens* phage jIBB-PF7A. Virol. J. 8, 142-147.

Sillankorva, S., Neubauer, P., Azeredo, J., 2008. Isolation and characterization of a T7-like lytic phage for *Pseudomonas fluorescens*. BMC Biotechnol. 8, 80-91.

Sillankorva, S., Neubauer, P., Azeredo, J., 2010. Phage control of dual species biofilms of *Pseudomonas fluorescens* and *Staphylococcus lentus*. Biofouling 26, 567-575.

Smith, D.E., Tans, S.J., Smith, S.B., Grimes, S., Anderson, D.L., Bustamante, C., 2001. The bacteriophage φ29 portal motor can package DNA against a large internal force. Nature 413, 748-752.

Sorhaug, T., Stepaniak, L., 1997. Psychrotrophs and their enzymes in milk and dairy products: Quality aspects. Trends Food Sci. Tech. 8, 35-41.

Sulakvelidze, A., Morris, J.G.Jr., Alavidze, Z., Pasternack, G.R., Brown, T.C., 2004. Method and device for sanitation using bacteriophages. [US 2004/0029250 A1] Ref Type: Patent.

Sulakvelidze, A., Sozhamamnnan, S., Pasternack, G.R., 2010. *Salmonella* bacteriophage and uses thereof. [US 7,674,467] Ref Type: Patent.

Suttle, C.A., 2005. Viruses in the sea. Nature 437, 356-361.

Tamanini, R., Beloti, V., Ribeiro Junior, J.C., Silva, L.C.C., Yamada, A.K., Silva, F.A., 2011. Contribuição ao Estudo da Qualidade Microbiológica e Físico-Química do Leite UHT. Revista Institucional do Laticínio "Cândido Tostes" 382, 27-33.

Ulitzur, S., Kuhn, J., 2000. Construction of lux bacteriophages and the determination of specific bacteria and their antibiotic sensitivities. Method Enzymol. 305, 543-557.

Wiedmann, M., Weilmeier, D., Dineen, S.S., Ralyea, R., Boor, J.K., 2000. Molecular and phenotypic characterization of *Pseudomonas* spp. isolated from milk. Appl. Environ. Microbiol. 66, 2085-2095.

Yoon, S., Kang, S., Kyoung, S., Choi, Y., Son, J., 2011. Bacteriophage having killing activity specific to *Staphylococcus aureus*. [US 8,071,352] Ref Type: Patent.

Yvon, M., Rijnen, L., 2001. Cheese flavour formation by amino acid catabolism. Int. Dairy J. 11, 185–201.

# 3. STRUCTURAL AND GENOMIC CHARACTERIZATION

# Complete genome sequence of the *Pseudomonas fluorescens* bacteriophage UFV-P2

*Abstract*

Milk proteolysis caused by *Pseudomonas fluorescens* is a serious problem in the dairy industries due to their ability to grow under refrigeration. The use of phages to control contaminants in food has been considered an alternative to traditional methods; therefore, thorough understanding of such organisms is vital for their use. In the present study, we show the complete genome sequence and analysis of a *P. fluorescens* phage isolated from waste water of a dairy industry in Brazil.

*Article*

*Pseudomonas fluorescens* is the major microorganism associated with milk deterioration by proteolysis (1, 2, 3). These bacteria produce heat-stable proteases and lipases responsible for the gelation of UHT milk and destabilization of the casein micelles and are associated with off-flavors and yield loss of dairy products (2, 3, 4, 5). Because the use of antibiotics and other broad-range antimicrobial agents can be discouraged due the need to maintain milk indigenous microbiota, the control of milk proteolysis using specific bacteriophages has been suggested as a strategic alternative (6, 7). However, its use on food would only be considered after a thorough

examination to ensure its safety and effectiveness. Therefore, it was essential to determine the complete genome sequence of phage UFV-P2, a *P. fluorescens* phage with a high ability to reduce casein proteolysis in milk.

Phage UFV-P2 was isolated and purified from the waste water of a dairy industry in Brazil, and then its genomic DNA was extracted and sequenced in Illumina Genome Analyzer II by CD Genomics (New York, USA). The viral genome was assembled and analyzed using CLC Genomics Workbench version 5.1 (CLCBio). The reads were assembled in contigs that considered more stringent parameters, in which 90% of each read had to cover the other read with 90% identity. This assembly produced the UFV-P2 genome sequence with coverage of 30,655-fold. Around 92 open reading frames (ORFs) were predicted using the Bacterial Genetic Code (NCBI translation table 11) and alternatives start codons (AUG,CUG, and UUG). All predicted ORFs were functionally annotated using Blastx searches against GenBank (http://www.ncbi.nlm.nih.gov/genbank) and UniProt (http://www.uniprot.org) databases. Only 41 ORFs (44.57%) presented significant similarities to known proteins and were considered in genome annotation. Additionally, the presence of tRNA genes was predicted using tRNAscan-SE program version 1.21 (8).

The phage UFV-P2 has a linear 45,517 bp DNA genome with no tRNA genes, a GC content of 51.5%, and 41 ORFs (19 positive- and 22 negative-stranded), representing a gene density of 0.9008/kb. The ORFs analyzed were annotated to five different protein groups, one of them containing 14 hypothetical proteins with unknown function (34.1%). The remaining groups consisted of one chaperone, four constitutive, and seven structural protein genes, including a major head, a portal, and a hypothetical tail collar domain.

Finally, 15 ORFs (36.6%) hits with genes encoding enzymes, including one lysozyme, the terminase small and large subunits, a exonuclease, a endonuclease, a primase/helicase and two parts of the DNA polymerase. The bioinformatics analyses showed 53.61% of identity with the genome of the temperate *Pseudomonas* phage PaP3 (9). Knowledge about this group of phages is still limited, and further analyses are needed to confirm UFV-P2's safety and its potential as an agent for biocontrol of milk contaminants.

**Nucleotide Sequence Accession Number.** The complete genome sequence of *P. fluorescens* phage UFV-P2 is available in GenBank under accession number JX863101.

*Acknowledgments*

We would like to thank to Cristina Dantas Vanetti, of the Federal University of Viçosa, for providing materials and technical support. This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of this manuscript.

*References*

1. **Arcuri EF, Silva PDL, Brito MAVP, Brito JRF, Lange CC, Magalhães MMA.** 2008. Counts, isolation and characterization of psychrotrophic bacteria from refrigerated raw milk. Ciência Rural **38**:2250-2255.

2. **Mu Z, Du M, Bai Y.** 2009. Purification and properties of a heat-stable enzyme of *Pseudomonas fluorescens* Rm12 from raw milk. Eur. Food Res. Technol. **228**:725-734.
3. **Nörnberg MFBL, Friedrich RSC, Weiss RDN, Tondo EC, Brandelli A.** 2009. Proteolytic activity among psychrotrophic bacteria isolated from refrigerated raw milk. Int. J. Dairy Technol. **63**:41-46.
4. **Dufour, D, Nicodeme M, Perrin C, Driou A, Brusseaux E, Humbert G, Gaillard JL, Dary A.** 2008. Molecular typing of industrial strains of *Pseudomonas* spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. Int. J. Food Microbiol. **125**:188-196.
5. **Kives J, Guadarrama D, Orgaz B, Rivera-Sem A, Vazquez J, SanJose C.** 2005. Interactions in biofilms of *Lactococcus lactis* ssp. *cremoris* and *Pseudomonas fluorescens* cultured in cold UHT milk. J. Dairy Sci. **88**:4165-4171.
6. **Karl T.** 2004. Old dogma, new tricks—21st century phage therapy. Nat. Biotechnol. **22**:31-36.
7. **Dixon B.** 2004. New dawn for phage therapy. Lancet Infect. Dis. **4**:186.
8. **Lowe TM and Eddy SR.** 1997. tRNAscan-SE: A program for improved detection of transfer RNA genes in genomic sequence. Nucleic Acids Res. **25**:955-964.
9. **Tan Y, Zhang K, Rao X, Jin X, Huang J, Zhu J, Chen Z, Hu X, Shen X, Wang L, Hu F.** 2007. Whole genome sequencing of a novel temperate bacteriophage of *P. aeruginosa*: evidence of tRNA gene mediating integration of the phage genome into the host bacterial chromosome. Cellular Microbiol. **9**:479–491.

# Genome annotation of the *Pseudomonas* phage UFV-P2: a new member of "LUZ24-like viruses"

*Abstract*

Phages infecting spoilage microorganisms have been considered as alternative biocontrol agents, and the study of their genomes is essential to their safe use in foods. UFV-P2 is a new *Pseudomonas fluorescens*-specific phage that has been tested for its ability to inhibit milk proteolysis. It belongs to the *Podoviridae* family and has a dsDNA genome of 45,517 bp, which contains at least 41 ORFs and a genome organization similar to the MR299-2, PaP3 and LUZ24 phage genomes, recently grouped as LUZ24-like viruses. In the present study, the structural genome analysis and the comparison of phylogenetic hypotheses lead us to propose the classification of φUFV-P2 in the LUZ24-like genus. Additionally, we propose the inclusion of the previously unclassified φtf in this genus.

*Introduction*

According to ICTV classification (http://www.ictvonline.org/virusTaxonomy.asp), three different genera of *Pseudomonas*-infecting *Podoviridae* can be distinguished. PhiKMV-like and T7-like genera belong to the subfamily *Autographivirinae*, and the LUZ24-like genus is in the *Picovirinae* subfamily, comprehending the viruses PaP3,

LUZ24 (8) and MR299-2 (1). Two unassigned species, the phages 119X (17) and F166 (6), are also classified in *Podoviridae*. This classification is based on biological characteristics and genome organization. Additionally, a search in the current databases showed that *Pseudomonas*-infecting *Podoviridae* still comprehends the genus of N4-like viruses with the phages LIT1 and LUZ7 (7) and others unclassified phages, including Bf7 (21), tf (16), and PaP2 (NC_005884).

Recently, we reported the genome announcement of *Pseudomonas fluorescens* bacteriophage UFV-P2 (12), a phage with a high ability to reduce casein proteolysis in milk. Milk proteolysis caused by thermoresistant enzymes produced by psychrotrophics is responsible for serious losses in the dairy industry due to negative effects on the quality and reduced shelf life of dairy products. In this environment, *Pseudomonas* spp. are prevalent contaminants (3, 18, 20), mainly *P. fluorescens* (2, 9). The use of phages in biocontrol has been suggested as an alternative to the use of chemicals (4, 23, 24), but they must be used with caution. In addition of proteolysis and biofilm inhibition studies and their host range definitions, it is necessary to understand phages' genome and proteome to make possible their use as biocontrol agents.

To expand our understanding about the *P. fluorescens*-specific phage UFV-P2 (12), we present in detail the analysis of its structural genome and comparisons to other phage genomes available in GenBank database.

## *Materials and methods*

### Sampling

The phage UFV-P2 was isolated from wastewater of a dairy industry in Minas Gerais, Brazil, and propagated in a strain of *P. fluorescens* 07A, courtesy of Laboratory of Food Microbiology, located at Federal University of Viçosa, Brazil, at 30 °C in LB medium.

Morphological analysis of UFV-P2

An aliquot of the viral extract (50 mL) was purified with 10 % PEG 8000 and used for electron microscopy studies. Ten microliters of a 10X diluted viral suspension was added to a 200-mesh grid that was covered with Formvar® for 5 min. The excess liquid was removed with filter paper, and the reaction was covered with 10 µl of 2 % uranyl acetate for 20 sec. The samples were visualized with a transmission electron microscope (Zeiss EM 109 TEM) operating at 80 kV at the Nucleus of Microscopy and Microanalysis (NMM) at UFV.

Genome extraction and composition

An amount of 500 µL of each phage solution was added of 75 µg/mL of proteinase K and 0.01% SDS and incubated at 56 °C for 90 min for disassembly of viral capsids. Proteins were removed using steps of centrifugations with phenol, phenol:chloroform (1:1), and chloroform. Genetic material was concentrated with an equal volume of isopropanol and resuspended in 30 µL of distillated water. For analysis of viral genome composition, 5 µL of the genomic extracts were submitted to restriction assays with enzymes DNase I (50 µg/mL) or RNase A (100 µg/mL) for 60 min at 37 °C. Extraction and restriction products were stained with GelRed (Biotium, USA) and visualized in 1% agarose gels.

Genomic DNA sequencing and assembly

UFV-P2 genome was extracted and sequenced in Illumina Genome Analyzer II by CD Genomics (New York, USA) and was assembled and analyzed using CLC Genomics Workbench version 5.1 (CLCBio). The sequenced reads were assembled in contigs considering more stringent parameters, in which 90% of each read had to cover the other read with 90% identity. The data from initial analyses, including genome assembly and ORFs prediction, were submitted by Eller et al. (12) and are available in GenBank database under accession number JX863101.

Bioinformatics analysis

To detect homologous proteins and functionally annotate each predicted ORF, BLASTX searches were carried out against GenBank (http://blast.ncbi.nlm.nih.gov) and UniProt (http://www.uniprot.org/blast) databases. Promoter sequences were predicted by BPROM Prediction of bacterial promoters (http://linux1.softberry.com/berry.phtml) and BDGP Neural Network Promoter Prediction (http://www.fruitfly.org/seq_tools/promoter.html). Transcriptional terminators were predicted by FindTerm (http://linux1.softberry.com/berry.phtml) and RibEx Riboswitch Explorer (http://132.248.32.45/cgi-bin/ribex.cgi). Putative tRNA-genes were analyzed by tRNA tRNAscan-SE (http://selab.janelia.org/tRNAscan-SE/). Additionally, a search for direct terminal repeats (DTRs) was carried out using Pygram (10).

For genomic comparisons purposes, genomic reference sequences (RefSeq) of possibly related phages were downloaded from GenBank (Table 4-1). These genomes were aligned using ClutalW (27), and a pairwise distance

matrix was calculated using CLC Genomics Workbench version 5.1 (CLCBio). Dot plot analysis was carried out using Nucleic Acid Dot Plots (http://www.vivo.colostate.edu/molkit/dnadot/index.html), considering a window size of 13 and a mismatch limit of 0.

Table 3-1. Comparison of *Pseudomonas* φUFV-P2 and others phage genomes.

| | | | | Phage UFV-P2 | |
|---|---|---|---|---|---|
| **Phage** | **GenBank Accession** | **GC content (%)** | **Genome density (genes/kbp)** | **Identities** | **%** |
| **UFV-P2** | JX863101 | 51.5 | 0.90* | - | - |
| **MR299-2** | JN254801 | 52.0 | 1.52 | 26,342 | 53.95 |
| **PaP3** | NC_004466 | 52.2 | 1.56 | 26,367 | 53.34 |
| **LUZ24** | NC_010325 | 52.2 | 1.49 | 26,196 | 53.01 |
| **Tf** | NC_017971 | 53.2 | 1.51 | 23,605 | 47.23 |
| **119X** | NC_007807 | 44.9 | 1.29 | 14,990 | 29.06 |
| **PaP2** | NC_005884 | 45.4 | 1.32 | 15,038 | 28.97 |
| **phiIBB-PF7A** | NC_015264 | 56.3 | 1.27 | 14,508 | 28.84 |
| **T7 (*Enterobacteria*)** | NC_001604 | 48.4 | 1.50 | 14,310 | 28.60 |
| **Phi-2** | NC_013638 | 58.9 | 1.00 | 14,803 | 28.40 |
| **phiKMV** | NC_005045 | 62.3 | 1.13 | 14,637 | 28.19 |
| **gh-1** | NC_004665 | 57.4 | 1.12 | 13,287 | 26.87 |
| **Bf7** | NC_016764 | 58.4 | 1.15 | 13,850 | 26.75 |
| **F116** | NC_006552 | 63.2 | 1.07 | 16,691 | 24.54 |
| **LUZ7** | NC_013691 | 53.2 | 1.54 | 18,541 | 24.38 |

*Considering only annotated coding sequences.

Phylogenetic trees

For clustering UFV-P2 phage in an evolutionary way, phylogenetic hypotheses were inferred by Bayesian inference (BI) and maximum likelihood (ML) using MrBayes v3.1.2 (15) and GARLI 2.0 (28), respectively.

Coding sequences (CDS) of major head protein (MH), lysozyme (LYS), terminase large subunit (TERM), and DNA polymerase part I (DNAP) were selected in the UFV-phage genome. Homologous CDS of these proteins were also selected in genomic reference sequences (RefSeq) of related phages (see Table 1). The codons of these four sequence sets (MH, LYS, TERM, and DNAP) were aligned using MUSCLE v.3.8.31 9 (11), a local alignment algorithm. Alignments were manually inspected, and the sites with gaps were excluded. To expedite the construction of phylogenetic trees, a model of nucleotide substitution was estimated using the jModeltest program (19). The GTR + I + G substitution model was selected as the best DNA evolution model for MH sequence set, the TIM2+I+G for LYS and TERM, and the TPM2uf+G for DNAP, according to the Akaike Information Criterion (AIC).

The BI phylogenetic trees were calculated using the Bayesian Markov Chain Monte Carlo (MCMC) method, in two runs with 50,000,000 generations and a sample frequency of 1,000. At the end of each run, the average standard deviations of the split frequencies were 0.005744 (MH sequence set), 0.004553 (LYS), 0.003808 (TERM), and 0.004084 (DNAP). The convergence of the parameters was analyzed in TRACER v1.5.0 (http://beast.bio.ed.ac.uk/tracer), and the chains reached a stationary distribution after 500,000 generations for all sequences sets. Then, a total of 1% of the generated trees was burned to produce the four consensus trees.

The respective substitution models of each sequence set were selected in the GARLI settings, and the statistical support of the ML phylogenetic trees was calculated by 1,000 bootstrap replicates. The 50% majority rule consensus trees of all bootstrap replicates was summarized using the SumTrees of DendroPy 3.8.0 (25).

## *Results and discussion*

### Isolation and morphology

The lysis plaques obtained from the isolation process were turbid and had wider diameters, indicating that UFV-P2 is a temperate phage. Transmission electron microscopy showed that phage UFV-P2 virions have isometric capsids of 40 to 50 nm in diameter and very short tails (see Figure 3-1), with morphological similarity to the *P. aeruginosa* phages Pap3 and MR299-2. UFV-P2 can be inserted in the *Podoviridae* family, order *Caudovirales*, in which are already included the *P. fluorescens* phages phiIBB-PF7A (22) and phi-2 (5).

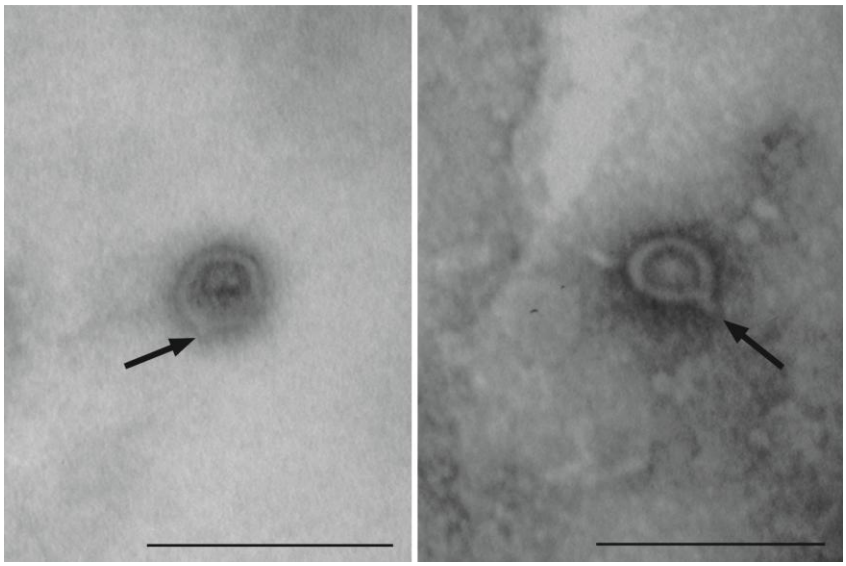


Figure 3-1. Transmission Electron Microscopy of the φUFV-P2. Virions have isometric capsids of 40-50 nm and very short tails (arrows). Scale bars = 100 nm.

Functional genomic organization

The viral genome was extracted, and different aliquots were digested with DNase I or RNase A. While DNAse I digestion degraded the viral genome, RNase A digestion had no effect, indicating that it was composed of DNA (data not shown). The phage UFV-P2 has a linear 45,517 bp DNA genome with a GC content of 51.5%, no DTRs, and was sequenced with coverage of 30,655 fold (Table 3-1).

The bioinformatics analyses showed that the UFV-P2 genome has a bidirectional organization with 92 predicted open reading frames (ORFs) larger than 100 bp, but only 41 ORFs (44.75%) could be identified as coding sequence (CDS) by similarity searches against known proteins in the Genbank and UniProt databases (see Figure 3-2 and Table 3-2). Thus, the UFV-P2

genomic density can vary from 0.90 to 2.02 genes/kb. The other *Pseudomonas* phages documented so far present a genomic density ranging from 1.00 to 2.04/kb (Table 3-2). Consequently, it is possible that some "no hits" ORFs correspond to non-described CDS.

Table 3-2. Functional genomic annotation based of phage UFV-P2.

| ORF | Start | End | Length | Sense | Start codon | Product | Similarity (GenBank access) | E-value* |
|---|---|---|---|---|---|---|---|---|
| **1** | 673 | 1146 | 474 | positive | ATG | terminase small subunit | *Pseudomonas* phage tf (YP_006382530) | 7,00E-63 |
| **2** | 1040 | 1573 | 534 | positive | CTG | lysozyme | *Pseudomonas* phage tf (YP_006382529) | 2,00E-53 |
| **3** | 1577 | 3022 | 1446 | positive | ATG | terminase large subunit | *Pseudomonas* phage LUZ24 (YP_001671939) | 0,00E+00 |
| **4** | 3001 | 5139 | 2139 | positive | TTG | portal protein | *Pseudomonas* phage MR299-2 (AFD10682) | 0,00E+00 |
| **5** | 5380 | 6366 | 987 | positive | ATG | Scaffolding protein | *Pseudomonas* phage LUZ24 (YP_001671936) | 8,00E-100 |
| **6** | 6385 | 7338 | 954 | positive | ATG | major head protein | *Pseudomonas* phage PaP3 (NP_775251) | 0,00E+00 |
| **7** | 7384 | 7710 | 327 | positive | TTG | hypothetical protein | *Pseudomonas* phage MR299-2 (AFD10686) | 1,00E-48 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **8** | 7714 | 8343 | 630 | positive | ATG | phage particle protein | *Pseudomonas* phage LUZ24 (YP_001671933) | 1,00E-77 |
| **9** | 8192 | 8542 | 351 | positive | TTG | hypothetical protein | *Pseudomonas* phage LUZ24 (YP_001671932) | 2,00E-26 |
| **10** | 8764 | 9399 | 636 | positive | ATG | tail fiber protein | *Pseudomonas* phage tf (YP_006382516) | 4,00E-39 |
| **11** | 9407 | 10948 | 1542 | positive | ATG | phage particle protein | *Pseudomonas* phage tf (YP_006382515) | 0,00E+00 |
| **12** | 10894 | 11658 | 765 | positive | CTG | hypothetical protein | *Pseudomonas* phage LUZ24 (YP_001671928) | 5,00E-51 |
| **13** | 11592 | 12089 | 498 | positive | TTG | hypothetical protein | *Pseudomonas* phage LUZ24 (YP_001671927) | 2,00E-55 |
| **14** | 12070 | 13014 | 945 | positive | ATG | phage particle protein | *Pseudomonas* phage tf (YP_006382512) | 1,00E-85 |
| **15** | 13011 | 13415 | 405 | positive | ATG | phage particle protein | *Pseudomonas* phage LUZ24 (YP_001671925) | 9,00E-07 |
| **16** | 13417 | 15129 | 1713 | positive | ATG | phage particle protein | *Pseudomonas* phage LUZ24 (YP_001671924) | 2,00E-55 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **17** | 15135 | 18299 | 3165 | positive | ATG | phage particle protein | *Pseudomonas* phage LUZ24 (YP_001671923) | 0,00E+00 |
| **18** | 18310 | 19197 | 888 | positive | ATG | phage particle protein | *Pseudomonas* phage LUZ24 (YP_001671922) | 5,00E-142 |
| **19** | 19199 | 19555 | 357 | positive | ATG | hypothetical protein | *Pseudomonas* phage MR299-2 (AFD10699) | 2,00E-38 |
| **20** | 20456 | 21211 | 756 | negative | ATG | hypothetical protein | *Pseudomonas* phage LUZ24 (YP_001671917) | 5,00E-128 |
| **21** | 21177 | 21737 | 561 | negative | TTG | endonuclease | *Pseudomonas* phage tf (YP_006382505) | 9,00E-40 |
| **22** | 21445 | 22440 | 996 | negative | ATG | hypothetical protein | *Pseudomonas* phage tf (YP_006382504) | 3,00E-38 |
| **23** | 22415 | 23260 | 846 | negative | CTG | 5'-3' exonuclease | *Pseudomonas* phage PaP3 (NP_775229) | 2,00E-138 |
| **24** | 23507 | 23884 | 378 | negative | CTG | hypothetical protein | *Pseudomonas* phage tf (YP_006382502) | 3,00E-30 |
| **25** | 24023 | 24481 | 459 | negative | CTG | hypothetical protein | *Pseudomonas* phage LUZ24 (YP_001671911) | 2,00E-43 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **26** | 24450 | 25046 | 597 | negative | ATG | hypothetical protein: DNA-binding protein | *Pseudomonas* phage tf (YP_006382500 YP_001526518) | 1,00E-68 |
| **27** | 25113 | 26636 | 1524 | negative | CTG | DNA polymerase part II | *Pseudomonas* phage tf (YP_006382498) | 0,00E+00 |
| **28** | 28852 | 29406 | 555 | negative | TTG | DNA polymerase part I | *Pseudomonas* phage tf (YP_006382490) | 1,00E-73 |
| **29** | 29342 | 31198 | 1857 | negative | ATG | primase; helicase | *Pseudomonas* phage PaP3 (NP_775217) | 0,00E+00 |
| **30** | 31349 | 31789 | 441 | negative | ATG | AIG2 family protein | *Pseudomonas* phage tf (YP_006382487) | 1,00E-25 |
| **31** | 31737 | 32612 | 876 | negative | ATG | glutathione synthase; ribosomal protein S6 modification enzyme (glutaminyl transferase) | *Pseudomonas* phage PaP3 (NP_775214) | 6,00E-77 |
| **32** | 32974 | 34434 | 1461 | negative | ATG | glutamine amidotransferase | *Pseudomonas* phage tf (YP_006382482) | 7,00E-140 |
| **33** | 34447 | 34965 | 519 | negative | CTG | HNH endonuclease | *Enterobacteria* phage BA14 (YP_002003475) | 2,00E-18 |
| **34** | 34860 | 36017 | 1158 | negative | ATG | amidoligase | *Pseudomonas* phage tf (YP_006382481) | 2,00E-91 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **35** | 35993 | 36577 | 585 | negative | CTG | hypothetical protein | *Pseudomonas* phage PaP3 (NP_775210) | 1,00E-38 |
| **36** | 36550 | 37347 | 798 | negative | ATG | hypothetical protein: putative COOH.NH2 ligase-type 2 | *Pseudomonas* phage tf (YP_006382479) | 2,00E-110 |
| **37** | 38229 | 39143 | 915 | negative | TTG | hypothetical protein | *Pseudomonas* phage tf (YP_006382478) | 5,00E-04 |
| **38** | 39804 | 40652 | 849 | negative | CTG | Transposase fusion protein | *Burkholderia thailandensis* Bt4 (ZP_02389877) | 4,00E-46 |
| **39** | 41060 | 41458 | 399 | negative | ATG | hypothetical protein | *Pseudomonas* phage tf (YP_006382473) | 3,00E-13 |
| **40** | 43535 | 44071 | 537 | negative | CTG | hypothetical protein | *Pseudomonas* phage tf (YP_006382463) | 1,00E-35 |
| **41** | 44035 | 44448 | 414 | negative | CTG | hypothetical protein | *Pseudomonas* phage PaP3 (NP_955002) | 0,002 |

* Expected values obtained in BLASTX searches. The smaller the E Value, the more significant the alignment.

The searches for consensus sequences of bacterial promoters revealed the presence of 14 promoters (supplementary material). Promoter sequences were evaluated according to annotated ORFs, and those without biological sense were not considered. Among the predicted bacterial promoters, 10 were found in the negative strand initiating the transcription of ORFs that codify for early proteins, which is a common feature of viral genomes that need bacterial transcription factors to start their infection cycle. The 4 other promoters were located in late genes modules. These genes are usually transcribed by viral transcription factors

Two sequences of Rho-independent transcription terminators were predicted, both in the positive strand. The first is located after the gene encoding the major head protein and was predicted by the RibEx software. The second, predicted by the program FindTerm, is located in the end of the ORF19, which encodes a hypothetical protein. The low number of sequences of Rho-independent terminators compared to the number of predicted ORFs may be due to the existence of other types of terminators or the presence of transcriptional modules and the generation of polycistronic mRNAs.

The predicted UFV-P2 genes were functionally classified as its promoters, predicted order of transcription, and its possible functions.

Biosynthesis and DNA replication

Twenty-two genes (ORFs 20-41) involved in the biosynthesis and viral replication process were found in the UFV-P2 genome negative strand, named early genes (Figure 3-2). Among viral replication genes, ORF23 encodes a 5'-3' exonuclease; ORFs 27 and 28 encodes the two parts of DNA polymerase,

between which there is a large non-coding intergenic region (about 2,000 bp); and ORF29 encodes a primase/helicase.

In addition, ORF38 encodes a transposase fusion protein related to the processes of integration of viral DNA in the bacterial chromosome. This protein presents the conserved HflC Band 7 domain (CDD accession cd03405,E-value 1.40e-03). According the Conserved Domains Database (CDD), this group includes proteins that are components of a complex that regulates the decision between the lysogenic and lytic cycles growth during lambda phage infection. In BLASTX searches, this protein presented significant hits with several bacterial proteins, including a hypothetical SPFH domain/Band 7 family protein of *Pseudomonas aeruginosa* ATCC 700888 (GenBank accession EKA49278; E-value 6e-58) and a transposase fusion protein of *Burkholderia thailandensis* Bt4 (GenBank accession ZP_02389877; E-value 4e-46). We also observed the presence of two endonucleases encoded by ORF21 and ORF33, a HNH endonuclease, a group I homing endonuclease. As described by Hertveldt et al. (14), these enzymes may be related to the presence of introns in the UFV-P2 genome; this remains to be confirmed in further studies.

In addition, ORF30, ORF31, and ORF32 encode, respectively, an AIG2-like (avirulence induced gene) family protein (CDD accession cd06661; E-value 3.01e-09), a glutathione synthase, and a glutamine amidotransferase. According to the CDD, AIG2-like family proteins catalyze the formation of pyroglutamic acid from dipeptides containing gamma-glutamyl. The synthesis and metabolism of glutathione tie the gamma-glutamyl cycle to numerous cellular processes, including protein and DNA synthesis. Thus, these proteins may act on key functions of virus-host modulation. The other 11 proteins of

the early genes module are hypothetical proteins without information about their functions.

Virion assembly and host lysis

Nineteen genes (ORFs 1-19) related to composition and assembly of the viral particle, DNA packaging, and host lysis were found in the UFV-P2 genome positive strand, named late genes (Figure 3-2). Two transcriptional clusters were found based on predicted bacterial promoters and terminators. The first cluster is located in the initial part of the genome (ORFs 1-6), and the second module, starting immediately after the first, corresponds to ORFs 7-19. The first transcriptional cluster terminator is partially overlapped to the sequence of the second transcriptional cluster promoter (Figure 3-2).

In the first cluster, ORF1and ORF3 encode the small and large terminase subunits, respectively. Based on the alignment results (supplementary material), we also suggest the annotation of ORF1 of φPaP3 (NP_775257) and ORF1 of φMR299-2 (AFD10679) as the terminase small subunit. The terminase is one motor component that assists the translocation of viral genomic DNA to the inner of the capsid during packaging via ATP hydrolysis. There is an ongoing discussion about the role of terminase structure in determining the points for cleavage of the viral DNA, which would influence the entire viral genome organization (13). ORF2 encodes a lysozyme that is used in the process of host cell break through the lysis of the peptidoglycan layer. ORF4 encodes the structural portal protein; ORF6 encodes the major head protein; and ORF5 encodes a scaffolding protein, which is a chaperone possibly related to viral particle assembly.

In the second cluster, ORF8, ORF11, and ORFs 14-18 encode particle proteins; ORF10 encodes the tail fiber protein; and the other 5 ORFs encode hypothetical proteins.

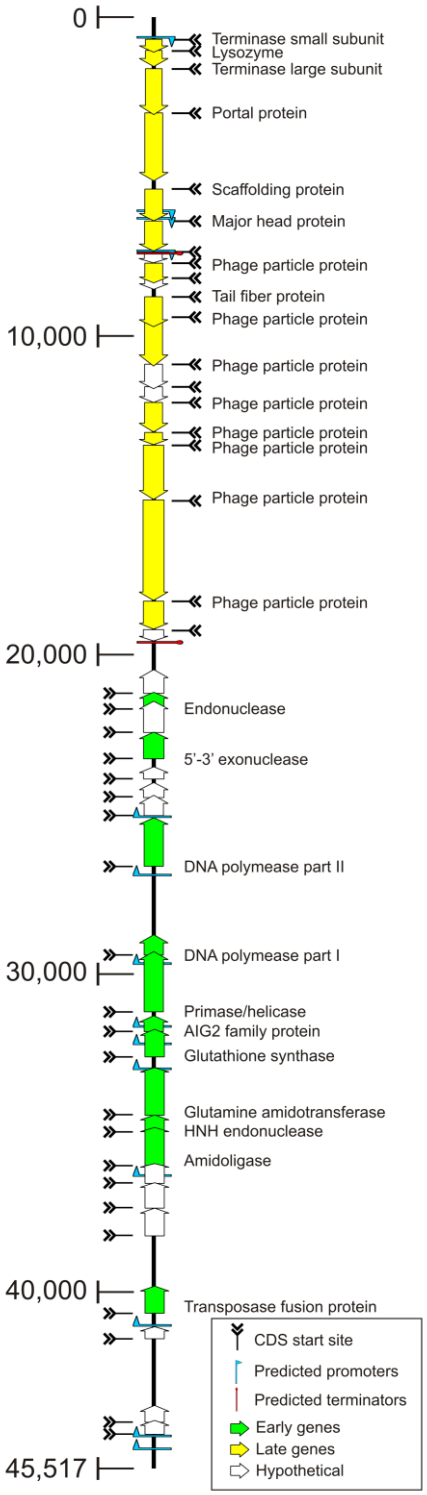



Figure 3-2. The φUFV-P2 genomic organization.

## Structural genomic comparisons and evolutionary clustering

The alignment of genomic sequences and pairwise comparisons revealed that MR299-2, PaP3, LUZ24, and tf are the most closely related phages to UFV-P2. Genomic sequences of these phages presented an identity to the UFV-P2 genome ranging from 47% to 53%, while to other phages genome sequences, it ranged from 24% to 29% (see Table 3-1).

The dot plot analysis showed a high co-linearity across the genomes of these phages, mainly in late genes (Figure 3-3). Phages MR299-2, PaP3, LUZ24, tf, and UFV-P2 present a conserved bidirectional genomic organization, which was reflected in the functional annotation of the UFV-P2 genome. Proteins of these 4 phages were the top hits to UFV-P2 sequences in BLASTX searches (see Table 3-2) and can collaborate each other's functional annotations. In addition to genomic comparisons, the searches for DTRs indicated the absence of patterns at the ends of the UFV-P2, PaP3, and MR299-2 genomes, unlike that observed in the phages LUZ24 and tf genomes. Another remarkable difference is the presence of genes codifying for tRNAs in the genomes of phages PaP3, LUZ24, and MR299-2, and the absence in the UFV-P2 and tf genomes.

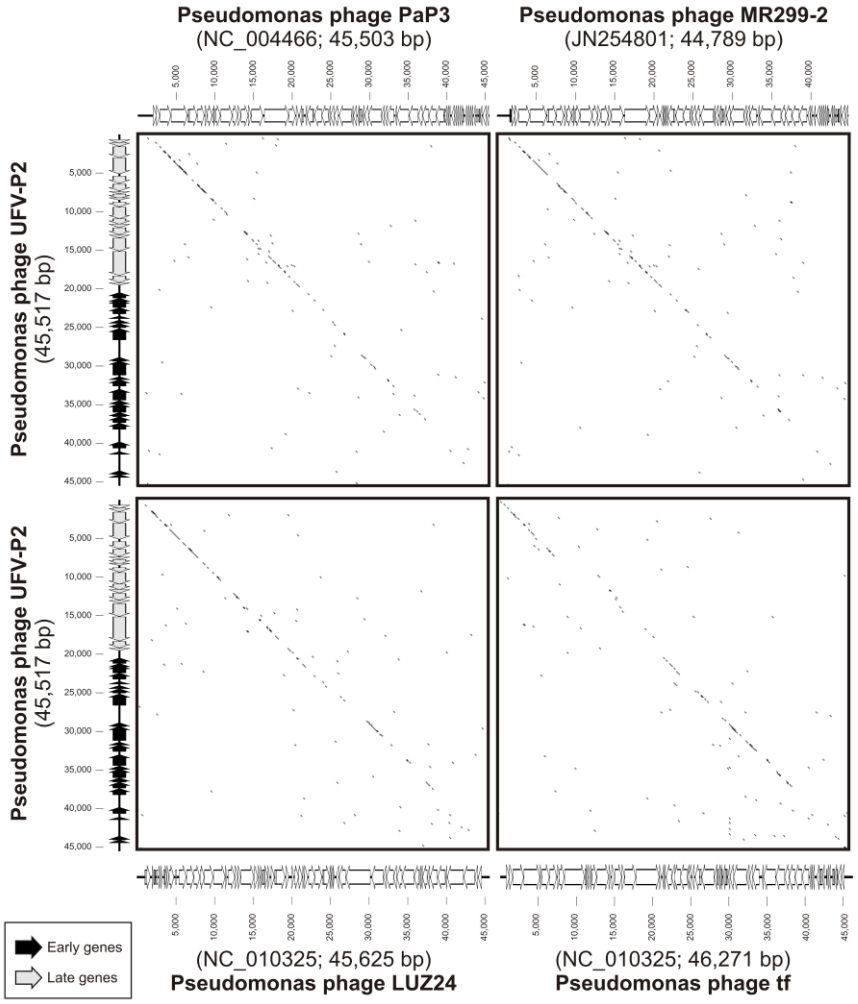



Figure 3-3. Dot plot alignment between the φUFV-P2 genome and the φPaP3, φMR299-2, φLUZ24 and φtf genomes.

As suggested by the genomic comparisons, phylogenetic analyses of coding sequences of major head protein, lysozyme, terminase large subunit, and DNA polymerase part I revealed that the phages MR299-2, PaP3, LUZ24, tf, and UFV-P2 are closely related (see Figure 3-4). In all phylogenetic trees, these phages were included in a distinct monophyletic clade supported by moderate values of posterior probability (PP) (Bayesian tree) and bootstrap value (BV) (ML tree): PP>85 and BV>50. This clade possibly represents the

LUZ24-like genus. Based on the evolutionary relationships, we propose the classification of phages UFV-P2 and tf as LUZ24-like viruses.

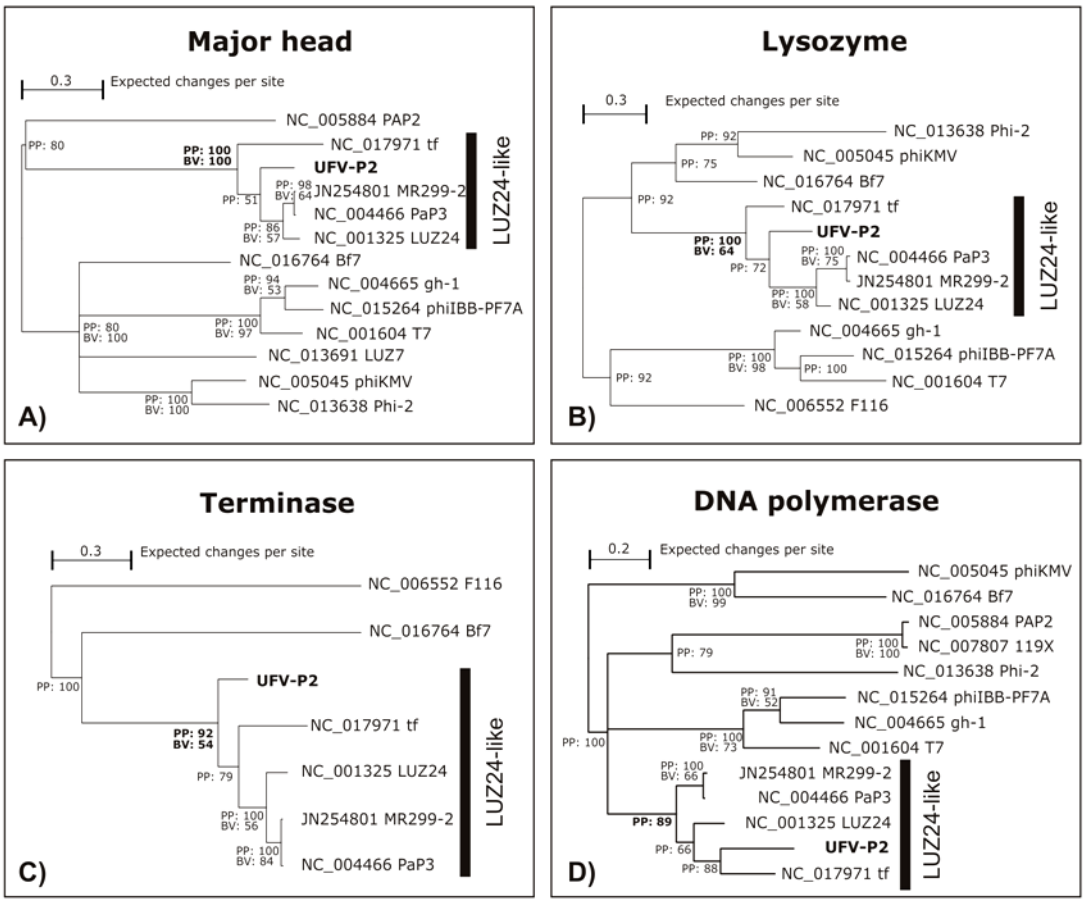



Figure 3-4. Evolutionary relationships between φUFV-P2 and other phages based on generally conserved proteins.

The majority rule consensus trees were obtained by Bayesian MCMC coalescent analysis of nucleotide coding sequences of A) Major head protein; D) Lysozyme; C) Terminase large subunit; D) DNA polymerase part I. The posterior probability values (PP) (expressed as percentages) calculated using the best trees found by MrBayes are shown beside each node. The second

value corresponds to bootstrap values (BV) (expressed as percentages) that define the clusters in the maximum likelihood tree.

Although not all phages have presented homologous sequences to analyzed coding sequences, we can observe that viruses of T7-like genus were also included in distinct monophyletic clades. On the other hand, it was not possible to define a phylogenetic clustering pattern to PhiKMV-like and the other unassigned viruses.

## *Conclusions*

We have presented the functional annotation of UFV-P2, a new *Pseudomonas fluorescens*-specific phage. Based on structural genomic comparison and phylogenetic analyses, we suggest the classification of UFV-P2 in the LUZ24-like genus. Additionally, we propose the inclusion of φtf, a previously unclassified phage, in this genus.

## *Acknowledgments*

This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of this manuscript.

*References*


1. **Alemayehu D, Casey PG, McAuliffe O, Guinane CM, Martin JG, Shanahan F, Coffey A, Ross RP, Hill C**. 2012. Bacteriophages φMR299-2 and φNH-4 can eliminate Pseudomonas aeruginosa in the murine lung and on cystic fibrosis lung airway cells. mBio **3**:e00029–12.

2. **Arcuri EF, Aparecida M, Paiva V, Lange CC**. 2008. Contagem , isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. Ciência Rural **38**:2250–2255.

3. **Baruzzi F, Lagonigro R, Quintieri L, Morea M, Caputo L**. 2012. Occurrence of non-lactic acid bacteria populations involved in protein hydrolysis of cold-stored high moisture Mozzarella cheese. Food microbiology **30**:37–44.

4. **Baum MM, Kainović A, O'Keeffe T, Pandita R, McDonald K, Wu S, Webster P**. 2009. Characterization of structures in biofilms formed by a Pseudomonas fluorescens isolated from soil. BMC microbiology **9**:103.

5. **Buckling A, Rainey PB**. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. Proceedings. Biological sciences / The Royal Society **269**:931–6.

6. **Byrne M, Kropinski AM**. 2005. The genome of the Pseudomonas aeruginosa generalized transducing bacteriophage F116. Gene **346**:187–94.

7. **Ceyssens P-J, Brabban A, Rogge L, Lewis MS, Pickard D, Goulding D, Dougan G, Noben J-P, Kropinski A, Kutter E, Lavigne R**. 2010. Molecular and physiological analysis of three Pseudomonas aeruginosa phages belonging to the "N4-like viruses". Virology **405**:26–30.

8. **Ceyssens P-J, Hertveldt K, Ackermann H-W, Noben J-P, Demeke M, Volckaert G, Lavigne R**. 2008. The intron-containing genome of the lytic Pseudomonas phage LUZ24 resembles the temperate phage PaP3. Virology **377**:233–8.

9. **Dogan B, Boor KJ**. 2003. Genetic diversity and spoilage potentials among Pseudomonas spp. isolated from fluid milk products and dairy processing plants. Applied and environmental microbiology **69**:130–8.

10. **Durand P, Mahé F, Valin A-S, Nicolas J**. 2006. Browsing repeats in genomes: Pygram and an application to non-coding region analysis. BMC bioinformatics **7**:477.

11. **Edgar RC**. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic acids research **32**:1792–7.

12. **Eller MR, Salgado RL, Vidigal PMP, Alves MP, Dias RS, de Oliveira LL, da Silva CC, de Carvalho AF, de Paula SO**. 2013. Complete Genome Sequence of the Pseudomonas fluorescens temperate bacteriophage UFV-P2. Genome Announcement.

13. **Feiss M, Rao VB**. 2012. The bacteriophage DNA packaging machine. Advances in experimental medicine and biology **726**:489–509.

14. **Hertveldt K, Lavigne R, Pleteneva E, Sernova N, Kurochkina L, Korchevskii R, Robben J, Mesyanzhinov V, Krylov VN, Volckaert G**. 2005. Genome comparison of Pseudomonas aeruginosa large phages. Journal of molecular biology **354**:536–45.

15. **Huelsenbeck JP, Ronquist F**. 2001. MRBAYES: Bayesian inference of phylogenetic trees. Bioinformatics (Oxford, England) **17**:754–5.

16. **Kulakov LA, Kochetkov VV, Ksenzenko VN, Krylov VN, Boronin AM**. 1988. [Physical map of the DNA of bacteriophage tf of Pseudomonas putida]. Molekuliarnaia genetika, mikrobiologiia i virusologiia 12–6.

17. **Kwan T, Liu J, Dubow M, Gros P, Pelletier J**. 2006. Comparative genomic analysis of 18 Pseudomonas aeruginosa bacteriophages. Journal of bacteriology **188**:1184–7.

18. **Munsch-Alatossava P, Alatossava T**. 2006. Phenotypic characterization of raw milk-associated psychrotrophic bacteria. Microbiological research **161**:334–46.

19. **Posada D**. 2008. jModelTest: phylogenetic model averaging. Molecular biology and evolution **25**:1253–6.

20. **Rasolofo EA, St-Gelais D, LaPointe G, Roy D**. 2010. Molecular analysis of bacterial population structure and dynamics during cold storage of untreated and treated milk. International journal of food microbiology **138**:108–18.

21. **Sajben-Nagy E, Maróti G, Kredics L, Horváth B, Párducz A, Vágvölgyi C, Manczinger L**. 2012. Isolation of new Pseudomonas tolaasii bacteriophages and genomic investigation of the lytic phage BF7. FEMS microbiology letters **332**:162–9.

22. **Sillankorva S, Kluskens LD, Lingohr EJ, Kropinski AM, Neubauer P, Azeredo J**. 2011. Complete genome sequence of the lytic Pseudomonas fluorescens phage ϕIBB-PF7A. Virology journal **8**:142.

23. **Sillankorva S, Neubauer P, Azeredo J**. 2008. Isolation and characterization of a T7-like lytic phage for Pseudomonas fluorescens. BMC Biotechnology **8**:80.

24. **Sillankorva S, Neubauer P, Azeredo J**. 2010. Phage control of dual species biofilms of Pseudomonas fluorescens and Staphylococcus lentus. Biofouling **26**:567–75.

25. **Sukumaran J, Holder MT**. 2010. DendroPy: a Python library for phylogenetic computing. Bioinformatics (Oxford, England) **26**:1569–71.

26. **Tan Y, Zhang K, Rao X, Jin X, Huang J, Zhu J, Chen Z, Hu X, Shen X, Wang L, Hu F**. 2007. Whole genome sequencing of a novel temperate bacteriophage of P. aeruginosa: evidence of tRNA gene mediating integration of the phage genome into the host bacterial chromosome. Cellular microbiology **9**:479–91.

27. **Thompson JD, Higgins DG, Gibson TJ**. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic acids research **22**:4673–80.

28. **Zwickl D**. 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. The University of Texas, Austin, TX.

*Supplementary material*

Table 3-3. Promoters predicted by BPROM* (Prediction of bacterial promoters).

| Promoter Position | LDF | -10 box Position | -10 box score | -10 box sequence | -35 box Position | -35 box score | -35 box sequence |
|---|---|---|---|---|---|---|---|
| 646 | 7.73 | 631 | 84 | TGGTATACT | 611 | 66 | TTGACA |
| 6369 | 5.96 | 6354 | 58 | TGGTACATT | 6332 | 20 | CTGCAA |
| 7426 | 3.08 | 7411 | 26 | TCGTTTGAT | 7390 | 38 | TTGAGT |
| 25068 | 7.44 | 25083 | 68 | TGGTACACT | 25103 | 66 | TTGACA |
| 44868 | 5.72 | 44883 | 59 | CGCTAACAT | 44903 | 66 | TTGACA |
| 26882 | 3.81 | 26897 | 53 | GGGTGTAAT | 26918 | 52 | TTGATG |

*Available at http://linux1.softberry.com/berry.phtml?topic=bprom&group=programs&subgroup=gfindb.
Threshol for promoters prediction: 0.20.

Table 3-4. Promoters predicted by BDGP Neural Network Promoter Prediction*.

| Start Position | End Position | Score | Sequence** |
|---|---|---|---|
| 605 | 650 | 0.99 | TAGGGGTTGACAAAAGGTAATTCCTATGGTATACTATATC**T**ATAGTTTAA |
| 6050 | 6095 | 0.95 | AGGCTGATTGGGCAGAACGAATGTTCAAGTATGGTGCTAC**C**GTTGGCTAT |
| 7366 | 7411 | 0.97 | CTTTTTTGTTTTAGGAGATTGATTTTGAGTATTACTTCCG**C**TTATTCGTT |
| 44508 | 44463 | 0.95 | GGCTGGTTTAGATTGTTGGCTGGTACAACTAGGGGATAAC**C**AACGAGTCG |
| 41088 | 41043 | 0.98 | GAAGAAAGGGCTTGACAAGTACCTGAAAGTGTGATAAGAT**A**ACCTTAAGG |
| 36342 | 36297 | 0.99 | TGACCAACTGTTGGACTGGCGTCCGTTCAGTGGTTACTAT**G**ACATTGACG |
| 33007 | 32962 | 0.97 | GAGTTCCTTTGTGGTGAGTGCTGTGAAGAAGGAATGTAAT**C**ATGTCCCTA |
| 32195 | 32150 | 0.97 | AACTCGAAGTTGTCAAACGCATCCGTGACAACAACATCAT**C**CCAGTGTTC |
| 31687 | 31642 | 0.98 | ACCACACCTTGCTGGAAATGCATCCAGACGAAGTTAAGTT**C**TTGGGCAAG |
| 29686 | 29641 | 0.97 | ACATTGCAGGTTTGGCCGATGAGCTGAACATTCGTATCTT**C**ATCTTCTCT |
| 26921 | 26876 | 0.97 | GTAACGGTTGATGGGCAGTTGTATCAGAGGGTGTAATCAT**G**ACGGTATAT |
| 25110 | 25065 | 0.97 | TAAGAAAGTGCTTGACATTTTGTGTATCATGTGGTACACT**T**TTCCCTCGT |

*Available at http://www.fruitfly.org/seq_tools/promoter.html.

* * Marked position corresponds to transcription site start.

Minimum promoter score prediction (between 0 and 1): 0.95.

Table 3-5. BLASTX searches result of ORF1 nucleotide sequence (terminase small subunit) of *Pseudomonas* phage UFV-P2.

| **GenBank Accession** | **Organism** | **E-value** | **Description** |
|---|---|---|---|
| AFD10679 | *Pseudomonas* phage MR299-2 | 2e-64 | hypothetical protein |
| NP_775257 | *Pseudomonas* phage PaP3 | 5e-64 | hypothetical protein |
| YP_001671941 | *Pseudomonas* phage LUZ24 | 3e-63 | hypothetical protein |
| YP_006382530 | *Pseudomonas* phage tf | 7e-63 | terminase small subunit |

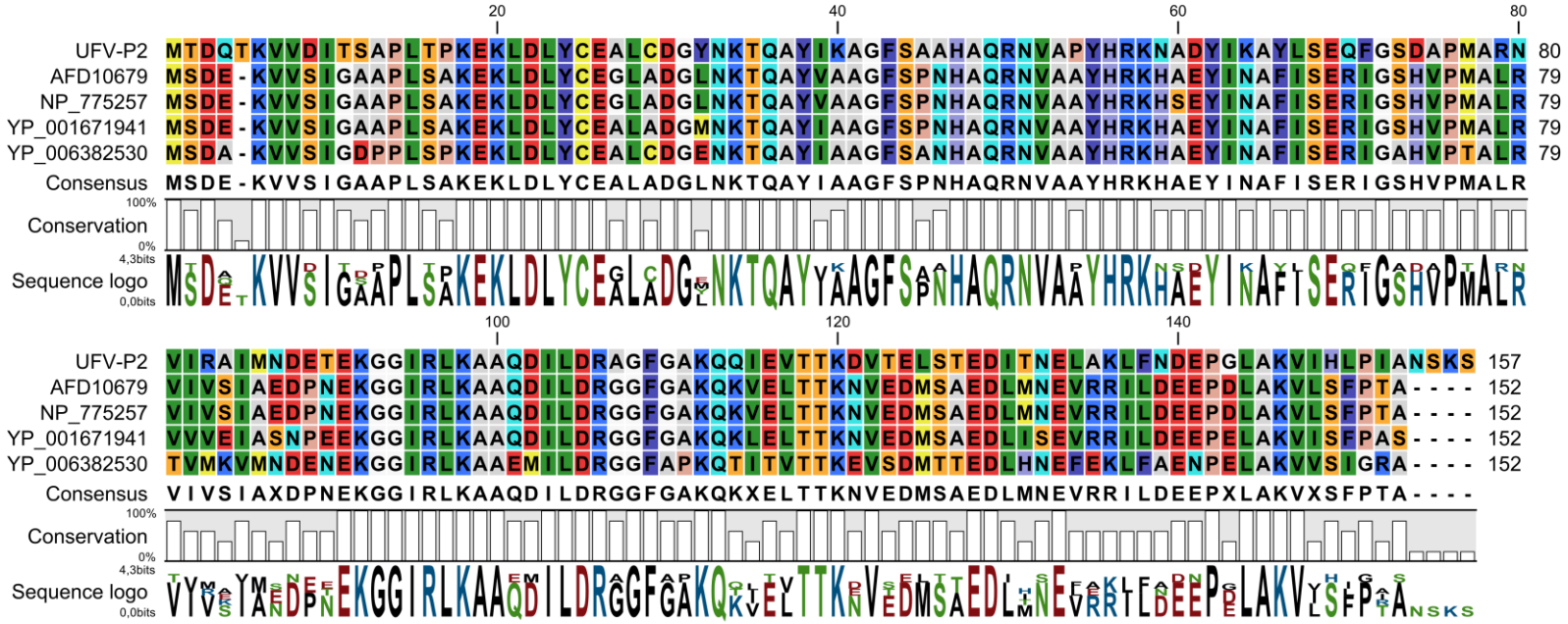



Figure 3-5. Terminase small subunit aminoacid sequences alignment of UFV-P2, MR299-2, PAP3, LUZ24, and tf phages.

# 4. CONTROL OF MILK PROTEOLYSIS BY THE PHAGE UFV-P2

## Control of milk proteolysis by the temperate *Pseudomonas* phage UFV-P2

*Abstract*

Milk proteolysis, primarily caused by *Pseudomonas fluorescens*, is a serious problem in the dairy industry because of refrigerated raw milk storage. In this study, the temperate *Pseudomonas* phage UFV-P2, previously isolated from waste water from a dairy plant, was concentrated using PEG and subjected to an electrophoresis in polyacrylamide gel. The pattern of structural proteins presented by UFV-P2 revealed at least 5 bands, which were associated to previously annotated ORFs, including the major head protein and the tail fiber protein, which are transcribed by the ORF6 and ORF10, respectively. The ability of this phage to inhibit proteolysis in reconstituted milk was tested, and the densitometric analysis of the casein bands showed that this phage reduced the proteolysis of these proteins during milk storage at 10 °C. However, the phage did not reduce the colony count in this environment and the mechanisms for which UFV-P2 inhibit milk proteolysis remains unknown. The phage UFV-P2 could represent a competitive candidate for controlling the gelation of UHT milk produced in the dairy industry.

*Keywords*: Biocontrol, *Pseudomonas fluorescens*, milk proteolysis.

*Introduction*

Raw milk storage and refrigeration at the production source was standardized in Brazil by the Ministry of Agriculture only recently, in 2002. The standardized practices reduce milk deterioration by acidifying activity of mesophilic bacteria (Cempirkova, 2002; Nicodème et al., 2005). However, refrigeration of raw milk still allows the growth of psychrotrophics microorganisms, which produce thermically-stable enzymes that are responsible for UHT milk gelification, as well as the destabilization of milk casein micelles. Moreover, these enzymes are associated with off-flavor formation and loss of yield in dairy production (Dufour et al., 2008; Kives et al., 2005; Mu et al., 2009; Nornberg et al., 2010). *Pseudomonas fluorescens* is the main psychrotrophic microorganism associated with milk deterioration because it exhibits significant proteolytic activity (Arcuri et al., 2008; Mu et al., 2009; Nornberg et al., 2010).

Studies involving phages as a way to control pathogens have increased due to appearance of antibiotic resistant bacteria, beyond the positive results of phage therapy in animals (Atterbury et al., 2003; Goode et al., 2003; Raya et al., 2006; Wagenaar et al., 2005). Bacteriophages possess antimicrobial activity similar to antibiotics. However, the use of phages has certain advantages over the use of antibiotics, such as host specificity (Thiel, 2004); their ability to exponentially multiply over time (Dixon, 2004) and the lack of collateral effects, because bacteriophages are inoffensive to mammal cells (Thiel, 2004; Yao and Moellering, 1995). The presence of phages in an industrial environment can be employed to limit pathogen propagation as well as reduce product contamination at different stages of production and processing (Sulakvelidze et al., 2004).

Previous studies reported the isolation and genomic characterization of the phage UFV-P2 (Eller et al., 2013a), which possess genomic similarity to the *Pseudomonas aeruginosa*-specific phages PaP3, LUZ24 and MR299-2 and to the *Pseudomonas putida*-specific phage tf (non published data), which are phages tested as agents for phage therapy. In this study, the phage UFV-P2 was tested for its ability to inhibit proteolysis in milk by a proteolytic strain of *P. fluorescens* isolated from refrigerated milk (Martins et al., 2005; Pinto, 2004).

## *Materials and methods*

### Strains and culture conditions

The strain *of P. fluorescens* 07A used in this study was supplied by the Laboratory of Food Microbiology at the Federal University of Viçosa, Brazil. The bacterium was stored in a buffer containing glycerol. It was activated in Luria-Bertani broth (LB, Sigma, USA) at 30°C for a period of 48 hours. This medium was used to culture and maintain the cells throughout the experiments.

The UFV-P2 phage solution was used to infect *P. fluorescens* 07A during their exponential phase of growth. Plating was performed as described by Hull (1977).

For phage propagation, the lysis plaques that resulted from plating were added to 10 mL of a solution containing the bacteria *P. fluorescens* 07A in their exponential phase of growth. This mixture was added to 10 mL of LB medium and 4 mL of a 175 µg/mL mitomicin solution (Sigma, USA), a lytic

cycle inducer. The erlenmeyer flasks were incubated at 30 °C for 24 hours. The solutions were centrifuged at 5,000 x g for 10 minutes and the titer of phages in the supernatants was measured using the semi-solid plating method.

UFV-P2 protein profile

An aliquot of 50 mL of the suspension containing the propagated phage was purified with 10% PEG 8000 and used for the protein analysis. The total phage protein content was analyzed with electrophoresis on a 12% polyacrylamide gel under denaturing conditions (SDS-PAGE). The purified and unpurified solutions containing phage proteins (15 µl) were loaded and electrophoresed at 190 V for 1 h. A control treatment comprising the uninfected *P. fluorescens* 07A was also purified with PEG and submitted to the electrophoretic analysis, along with the pure LB Broth used in all experimental steps. The PageRuler® Unstained Protein Marker (Fermentas) was used as a molecular size marker, and the gel was stained with 3% ammoniacal silver.

Proteolysis Assay

Four flasks containing 100 mL of sterile 12% Reconstituted Skim Milk (RSM) were inoculated with different treatments (Table 4-1) and incubated at 10 °C for 7 days. Ten milliliters aliquots were collected on days 0, 3, 5 and 7. They were acidified to pH 4.0, then centrifuged at 5,000 x g for 10 minutes to precipitate the casein. The supernatant (serum) was discarded and the pellets were stocked at -20 °C until day 7 of the assay, when they were resuspended to a final volume of 10 mL with 0.5 M Tris-HCl pH 6.8. Samples were submitted to a denaturing polyacrylamide gel electrophoresis (SDS-PAGE – Laemmli, 1970). They were stained with Coomassie Blue solution, and

revealed using a 50% ethanol and 12% acetic acid solution. Gels were densitometrically analyzed using Image J software. This enabled the evaluation of the AE-P2 phage efficiency to inhibit proteolysis caused by the bacteria. A count of colony-forming units was performed on collect days 0, 3, 5 and 7 using the drop plate method (Morton, 2001).

After the last day of incubation at 10 °C, the flasks were maintained at room temperature for 5 days in order to allow their visual observation, since certain alterations in proteolyzed milk can be visually noted. The assay experiments were carried out independently three times.

Table 4-1. Proteolysis assay treatments.

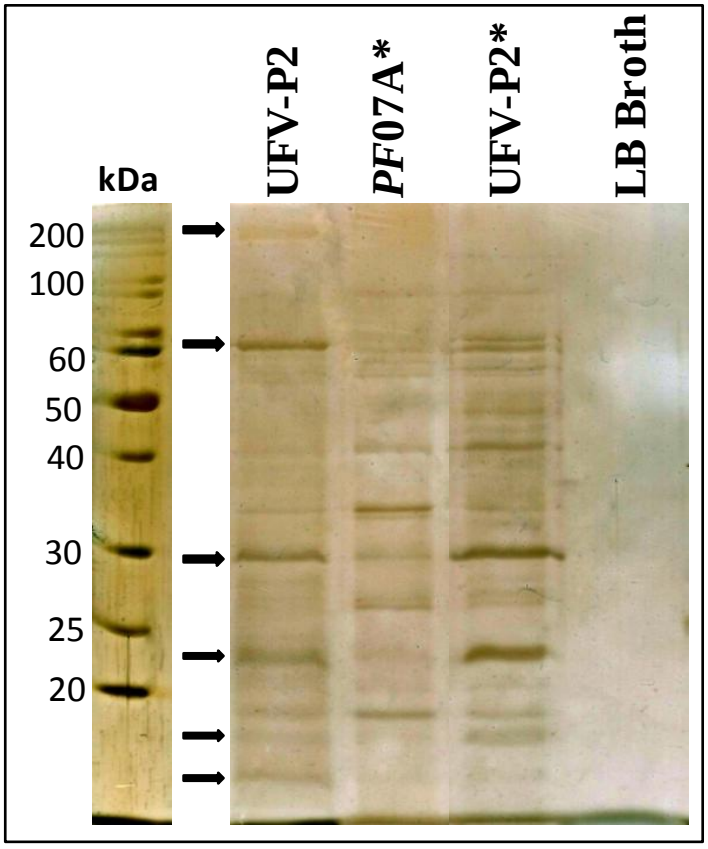
| Treatment | *Pseudomonas fluorescens* 07A $4{,}2x10^5$ CFU/mL | Phage UFV-P2 $10^7$ PFU/mL |
|---|---|---|
| 1 | - | - |
| 2 | + | - |
| 3 | - | + |
| 4 | + | + |

*Results*

UFV-P2 protein profile

The phages were purified using PEG and submitted to an electrophoresis under denaturizing conditions to verify the UFV-P2 pattern of proteins. Two control treatments were performed, one containing the non-infected bacteria purified with PEG (PF07A*), and one with LB Broth.

After staining using silver nitrate, it was not possible to observe any band in the column corresponding to the LB Broth, while control containing PF07* revealed a number of bands which were also present in the column corresponding to the phage UFV-P2* (Figure 4-1). At least 5 proteins were exclusive or substantially concentrated in the columns corresponding to the phage, whose molecular weights were about 60 (P60), 30 (P30), 23 (P23), and two under 20 kDa (arrows).




*Purified with PEG

Figure 4-1. Electrophoretic pattern of the UFV-P2 proteins.

Control of milk proteolysis

An aliquot of 10 mL of each treatment was collected on days 0, 3, 5 and 7 post-inoculation, and analyzed for the degree of proteolysis in a 12% polyacrylamide gel electrophoresis (Figure 4-2). The control treatment containing only the bacteria *P. fluorescens* 07A showed a high degree of proteolysis, since only 4.09, 6.08 and 0.01% of the total amount of α, β and κ-casein remained at 7 days post-inoculation, respectively (Figure 4-3). However, when the bacteria strain was inoculated with the phage, a decrease in the degree of casein degradation in the different fractions was observed as compared to the control treatment (Figures 4-2 and 4-3).

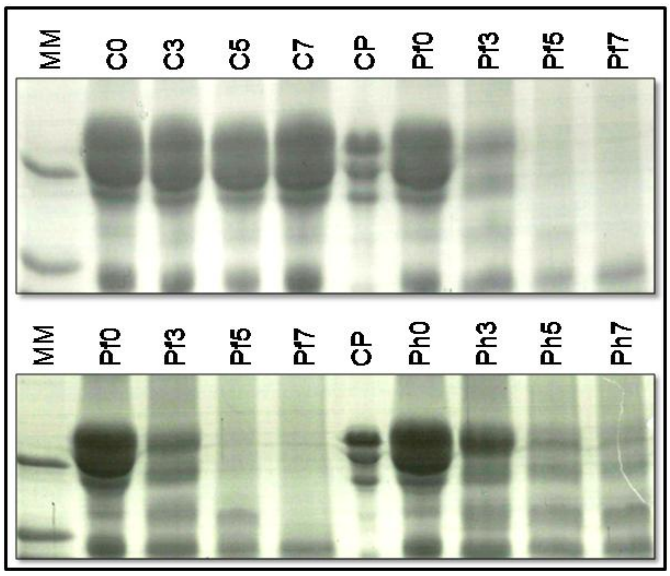



Figure 4-2. SDS-PAGE of the proteolysis assay. Profile of casein bands at 0, 3, 5 and 7 days after inoculation. Control treatments: MM: low-weight molecular marker, C: control of LDR 12% medium at days 0 (C0); 3 (C3); 5 (C5) and 7 (C7) pos-inoculation; CP: casein pattern; PF: control of LDR 12% medium inoculated with *Pseudomonas fluorescens* 07A at days 0 (PF0); 3 (PF3); 5 (PF5) and 7 (PF7) pos-inoculation. Proteolysis patterns comparison was performed between control with PF07A and treatment inoculated with bacteria and the UFV-P2 phage (Ph0, Ph3, Ph5 and Ph7).

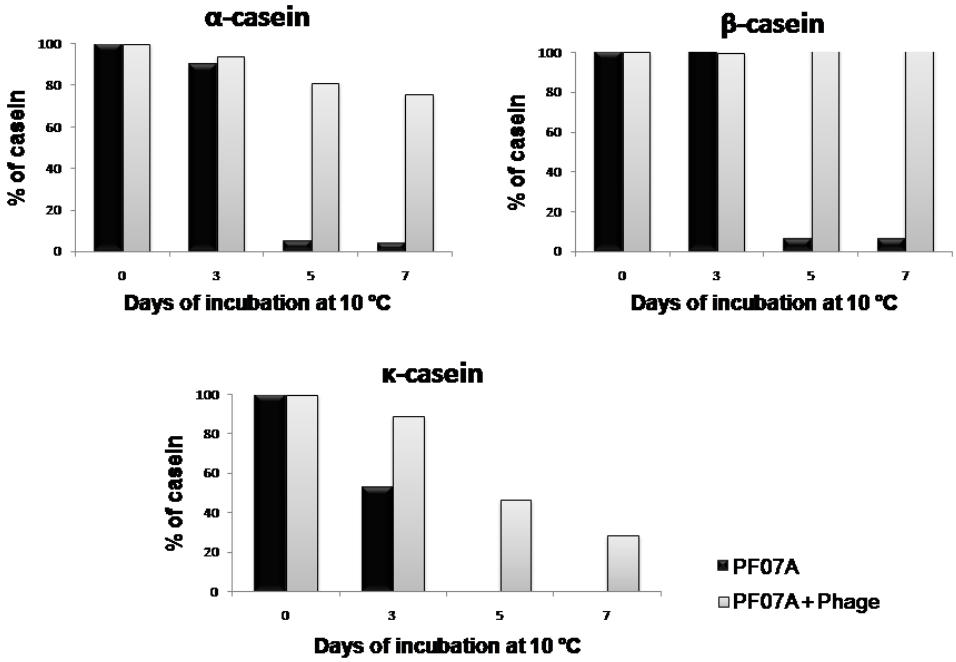



Figure 4-3**.** Densitometric analysis of the casein bands from the proteolysis assay. The UFV-P2 phage was able to reduce proteolysis in milk inoculated with *Pseudomonas fluorescens*. Treatment with bacteria presented a final quantity of 4.09, 6.08 and 0.01% of α, β and κ-casein, respectively (from up to down), while treatment containing bacteria with phage presented a final quantity of 76.02, 100.00 and 28.80% of these proteins.

Although the phage's presence showed inhibitory activity over proteolysis, no apparent reduction was observed in the number of colony-forming bacteria units on these treatments compared to the control treatment (data not shown).

All treatments were incubated at room temperature for 5 days after the end of the proteolysis assay period. Treatment 2 (T02), which contained only the bacteria, became heterogeneous, with two distinct phases. Its superior phase showed significant darkening and an aqueous appearance. Compared to

T02, treatment 4 (T04) containing bacteria plus the UFV-P2 phage, showed only minor color alterations and no phase separation (Figure 4-4).

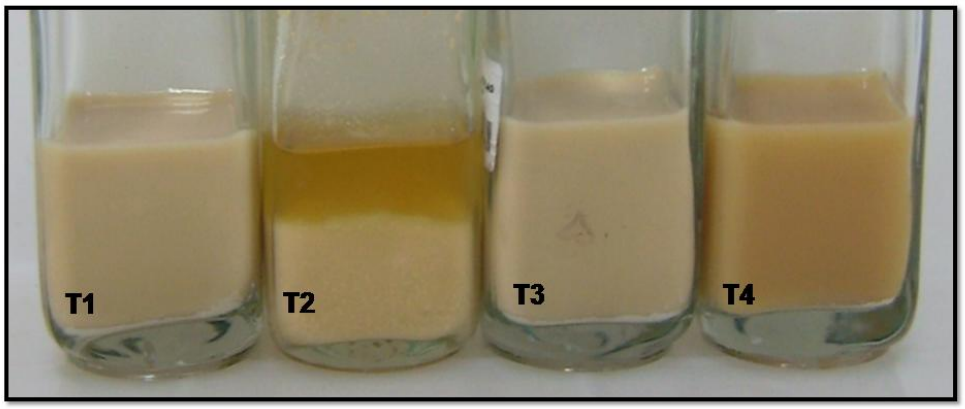



Figure 4-4. Visual effects of the treatments incubated at room temperature. Treatments from the proteolysis assay were incubated at room temperature during five days and the visual effects caused by the proteolytic enzymes could be observed. 1) Pure Reconstituted Skim Milk (RSM); 2) RSM + *Pseudomonas fluorescens* 07A; 4) RSM + UFV-P2 phage and 5) RSM + *Pseudomonas fluorescens* 07A + UFV-P2 phage.

*Discussion*

In this study, the phage UFV-P2, previously isolated from waste water of a dairy plant (Eller et al., 2013), showed to be efficient in slowing proteolysis in milk by a proteolytic strain of *P. fluorescens* (Martins et al., 2005; Pinto, 2004).

The electrophoretic pattern of viral proteins found in this study could be associated with the annotated ORFs in the UFV-P2 genome (Eller et al., 2013). Among the protein bands observed, at least six were considered as belonging to the phage, either by their exclusive presence either by their

higher concentration relative to column PF07A*. These bands have the approximate sizes of 150, 60, 30, 23, and two between 15 and 20 kDa (Fig. 4-1) and its correspondence with the annotated ORFs can be estimated by the relation: 1 amino acid = 110 Da (Krawetz and Womble, 2003). Since viral proteins that are sufficiently concentrated as to be observed on polyacrylamide gels are almost always structural components of the viral particle, the proteins visualized in this study probably correspond to the ORFs annotated in the first half of the UFV-P2 genome, corresponding to later transcribed genes.

Among the viral proteins, the generally most abundant is the viral capsid protein, or major head protein, whose gene has been annotated in the ORF6 in the phage UFV-P2. This ORF contains 954 nucleotides, thus corresponding to a protein of approximately 35 kDa. The presence of a protein highly concentrated at the height of 30 kDa suggests the presence of that protein. Another protein that is expected to be in great quantity in a electrophoretic pattern corresponds to the tail protein. According to the annotation performed by Eller et al (2013), the tail fiber protein is expressed by ORF10 and would correspond to a protein of approximately 23 kDa.

The P60, also concentrated in the gel, would corresponds to an ORF of about 1650 nucleotides. Analyzing the UFV-P2 annotated ORFs, it can be noticed the presence of at least 3 ORFs that could match this band: ORF3, which corresponds to the large terminase subunit (theoretical size of 53 kDa), ORF4, encoding the protein portal (theoretical size of 78 kDa), and ORF17, which encodes a protein particle similar to a protein of the phage LUZ24, whose function is still unknown (theoretical size of 63 kDa). The annotation for the bands appearing below 20 kDa is more difficult given the large amount of viral ORFs of consistent size and annotated as structural. All assumptions

and relationships discussed in this study need to be confirmed by further studies. An alternative could be the sequencing of the N-terminal region of each of these proteins.

The densitometric analysis of the casein bands on SDS-PAGE indicated that the phage UFV-P2 was able in reducing the degree of proteolysis in milk by *Pseudomonas*, although it did not reduce the initial population of this bacterium in the milk. This result may be due to the high initial population of cells inoculated ($4.2x10^5$). This parameter was chosen in order to optimize the production of proteolytic enzymes by *P. fluorescens*, which occurs at the end of the logarithmic phase of growth (Matselis and Roussis, 1998; Pinto et al., 2010). Nevertheless, various studies indicate that in the dairy industry, under adequate hygienic conditions, the initial population of psychrotrophics is not as high as the levels used in this study (Nornberg et al., 2010; Pinto et al., 2006). Thus, the presence of the phage UFV-P2 could be sufficient to inhibit milk proteolysis during the storage time until the milk is ready to be processed.

Additional studies may explain why the phage UFV-P2 was efficient in reducing proteolysis without a significant population decrease of *Pseudomonas*. A hypothesis is that the phage decreases enzyme production by bacteria, a process called “host shut-off”. Host shut-off acts by recruiting the cell protein synthesis machinery for its own protein expression and assembly, thus maintaining live, but inefficient bacterial cells. This can occur in several ways, such as the inhibition or reduction in the activity of host RNA polymerase through phosphorylation or binding of inhibitors, or the translational discrimination in favor of phage mRNA (Kruger and Schroeder, 1981).

The milk inoculated with the strain PF07A showed visible physical alterations, including phase separation and darkening of the superior phase. These alterations were caused by the high capacity of the proteolytic enzymes for degrading casein fractions (Adams et al., 1976), which ultimately caused milk destabilization and precipitate formation. The presence of the UFV-P2 lead to less severe alterations, with no observable phases. Thus it could be visually confirmed that UFV-P2 is efficient in slowing the milk deterioration process by *P. fluorescens* in the conditions of this study.

*Conclusions*

In this study, a *Pseudomonas* phage previously isolated from waste water in a dairy plant possess at least five main structural proteins ranging from 15 to 60 kDa, estimated by electrophoresis in polyacrylamide gel. These proteins include a major head and a tail fiber proteins and were theoretically associated to previously *in silico* annotated ORFs (Eller et al., 2013) according to their molecular sizes. Further studies are needed to confirm these statements.

The phage UFV-P2 was able to reduce α, β and κ-casein proteolysis in LDR 12% from 4.09, 6.08 and 0.01% of the initial amount, to 76.02, 100.00 and 28.80%, respectively, and to slow milk deterioration at room temperature. Further studies are needed to demonstrate the molecular mechanisms by which this occurs. The main hypothesis consists in the reduction of extracellular protease production in cells infected by the phage.

The phage UFV-P2 should be considered a potential agent for proteolysis control in the milk industry. Its uses can be applied at different stages of the

production process, as occurs with phages for the control of *Salmonella* and other pathogens in poultry farms (Atterbury et al., 2007; Higgins et al., 2005; Huff et al., 2006; Loc Carrillo et al., 2005). Studies to assess host specificity will also be performed in order to make the UFV-P2 phage available to the milk industry as soon as possible. This will allow it to be used to avoid further losses caused by contaminated proteolyzed milk.

*Acknowledgments*

We would like to thank to Cristina Dantas Vanetti of the Federal University of Viçosa, for providing materials and technical support. This study was supported by grants from the Fundação de Amparo à Pesquisa do Estado de Minas Gerais (FAPEMIG), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). The funders had no role in the study design, data collection, analysis, decision to publish, or preparation of this manuscript.

*References*

**Adams, D. M., J. T. Barach, and M. L. Speck**. 1976. Effect of psychrotrophic bacteria from raw milk on milk proteins and stability of milk proteins to ultrahigh temperature treatment. Journal of dairy science. Elsevier **59**:823-7.

**Ahiwale, S., N. Tamboli, K. Thorat, R. Kulkarni, H. Ackermann, and B. Kapadnis**. 2011. In vitro management of hospital Pseudomonas aeruginosa biofilm using indigenous T7-like lytic phage. Current microbiology **62**:335-40.

**Arcuri, E. F., M. Aparecida, V. Paiva, and C. C. Lange**. 2008. Contagem , isolamento e caracterização de bactérias psicrotróficas contaminantes de leite cru refrigerado. Ciência Rural **38**:2250-2255.

**Atterbury, R. J., M. a P. Van Bergen, F. Ortiz, M. a Lovell, J. a Harris, a De Boer, J. a Wagenaar, V. M. Allen, and P. a Barrow**. 2007. Bacteriophage therapy to reduce salmonella colonization of broiler chickens. Applied and environmental microbiology **73**:4543-9.

**Atterbury, R. J., P. L. Connerton, C. E. R. Dodd, C. E. D. Rees, and I. F. Connerton**. 2003. Application of Host-Specific Bacteriophages to the Surface of Chicken Skin Leads to a Reduction in Recovery of Campylobacter jejuni. Applied and environmental microbiology **69**:6302-6306.

**Buckling, A., and P. B. Rainey**. 2002. Antagonistic coevolution between a bacterium and a bacteriophage. Proceedings. Biological sciences / The Royal Society **269**:931-6.

**Cempirkova, R.** 2002. Psychrotrophic vs . total bacterial counts in bulk milk samples. VetMed Czech **2002**:227-233.

**Ceyssens, P.-J., K. Hertveldt, H.-W. Ackermann, J.-P. Noben, M. Demeke, G. Volckaert, and R. Lavigne.** 2008. The intron-containing genome of the lytic Pseudomonas phage LUZ24 resembles the temperate phage PaP3. Virology **377**:233–8.

**Cousin, M. A.** 1982. Presence and activity of psychrotrophic microrganisms in milk and dairy products: a review. Journal of Food Protection **45**:172-207.

**Dixon, B.** 2004. New dawn for phage therapy. The Lancet infectious diseases **4**:186.

**Dufour, D., M. Nicodème, C. Perrin, A. Driou, E. Brusseaux, G. Humbert, J.-L. Gaillard, and A. Dary**. 2008. Molecular typing of industrial strains of Pseudomonas spp. isolated from milk and genetical and biochemical characterization of an extracellular protease produced by one of them. International Journal of Food Microbiology **125**:188-96.

**Eller, M.R., R.L. Salgado, P.M.P. Vidigal, M.P. Alves, R.S. Dias, L.L. de Oliveira, C.C. da Silva, A.F. de Carvalho, and S.O. de Paula**. 2013a. Complete genome sequence of the *Pseudomonas fluorescens* bacteriophage UFV-P2. Genome Annotation **In press.**

**Eller, M.R., P.M.P. Vidigal, R.L. Salgado, M.P. Alves, R.S. Dias, L.L. de Oliveira, C.C. da Silva, A.F. de Carvalho, and S.O. de Paula**. 2013b. Genome annotation of the *Pseudomonas* phage UFV-P2: a new member of "LUZ24-like viruses". Journal of Virology **In press**.

**Goode, D., V. M. Allen, and P. A. Barrow**. 2003. Reduction of Experimental Salmonella and Campylobacter Contamination of Chicken Skin by Application of Lytic Bacteriophages. Society **69**:5032-5036.

**Higgins, J. P., S. E. Higgins, K. L. Guenther, W. Huff, a M. Donoghue, D. J. Donoghue, and B. M. Hargis**. 2005. Use of a specific bacteriophage treatment to reduce Salmonella in poultry products. Poultry science **84**:1141-5.

**Huff, W. E., G. R. Huff, N. C. Rath, and A. M. Donoghue**. 2006. Evaluation of the influence of bacteriophage titer on the treatment of colibacillosis in broiler chickens. Poultry Science **85**:1373-1377.

**Hull, R. R.** 1977. Methods for monitoring bacteriophage in cheese factories. Australian Journal of Dairy Technology **32**:63-64.

**Kives, J., D. Guadarrama, B. Orgaz, A. Rivera-Sen, J. Vazquez, and C. SanJose**. 2005. Interactions in biofilms of Lactococcus lactis ssp. cremoris and Pseudomonas fluorescens cultured in cold UHT milk. Journal of Dairy Science **88**:4165-4171.

**Krawetz S. A., and D. D. Womble.** 2003. Introduction to Bioinformatics: A Theoretical and Practical Approach. Human Press Inc., New Jersey, USA

**Krüger, D. H., and C. Schroeder**. 1981. Bacteriophage T3 and bacteriophage T7 virus-host cell interactions. Microbiological reviews **45**:9-51.

**Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. Nature Publishing Group **227**:680-685.

**Loc Carrillo, C., R. J. Atterbury, E. Dillon, A. Scott, I. F. Connerton, C. L. Carrillo, and P. L. Connerton**. 2005. Bacteriophage Therapy To Reduce Campylobacter jejuni Colonization of Broiler Chickens Bacteriophage Therapy To Reduce Campylobacter jejuni Colonization of Broiler Chickens. Applied and environmental microbiology **71**:6554-6563.

**Martins, M. L., E. F. De Araújo, H. C. Mantovani, C. A. Moraes, and M. C. D. Vanetti**. 2005. Detection of the apr gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology **102**:203-211.

**Martins, M. L., C. L. O. Pinto, R. B. Rocha, E. F. De Araújo, and M. C. D. Vanetti**. 2006. Genetic diversity of Gram-negative, proteolytic, psychrotrophic bacteria isolated from refrigerated raw milk. International Journal of Food Microbiology **111**:144-148.

**Matselis, E., and I. G. Roussis**. 1998. Proteinase and lipase production by Pseudomonas fluorescens. Proteolysis and lipolysis in thermized ewe's milk. Food Control **9**:251-259.

**Morton, R. D.** 2001. Aerobic plate count, pp. 63-67. *In* C. Vanderzant, and D.F. Splittsoesser (eds.), Compendium of methods for the microbiological examination of foods. APHA.

**Mu, Z., M. Du, and Y. Bai**. 2009. Purification and properties of a heat-stable enzyme of Pseudomonas fluorescens Rm12 from raw milk. European Food Research and Technology **228**:725-734.

**Nicodème, M., J.-P. Grill, G. Humbert, and J.-L. Gaillard**. 2005. Extracellular protease activity of different Pseudomonas strains: dependence of proteolytic activity on culture conditions. Journal of Applied Microbiology **99**:641-648.

**Nörnberg, M. F. B. L., R. S. C. Friedrich, R. D. N. Weiss, E. C. Tondo, and A. Brandelli**. 2010. Proteolytic activity among psychrotrophic

bacteria isolated from refrigerated raw milk. International Journal Of Dairy Technology **63**:41-46.

**Pinto, C. L. O.** 2004. Bactérias psicrotróficas proteolíticas do leite cru refrigerado granelizado destinado à produção do leite UHT. Tese apresentada à Universidade Federal de Viçosa para obtenção do título de Doutor em Microbiologia Agrícola, Departamento de Microbiologia.

**Pinto, C. L. O., M. L. Martins, and M. C. D. Vanetti**. 2006. Qualidade microbiológica de leite cru refrigerado e isolamento de bactérias psicrotróficas proteolíticas. Ciência e Tecnologia de Alimentos **26**:645-651.

**Pinto, U. M., E. D. Costa, H. C. Mantovani, and M. C. D. Vanetti**. 2010. The proteolytic activity of Pseudomonas fluorescens 07A isolated from milk is not regulated by quorum sensing signals. Brazilian Journal of Microbiology **41**:91-96.

**Raya, R. R., P. Varey, R. a Oot, M. R. Dyen, T. R. Callaway, T. S. Edrington, E. M. Kutter, and A. D. Brabban**. 2006. Isolation and characterization of a new T-even bacteriophage, CEV1, and determination of its potential to reduce Escherichia coli O157:H7 levels in sheep. Applied and environmental microbiology **72**:6405-10.

**Sillankorva, S., P. Neubauer, and J. Azeredo**. 2008. Isolation and characterization of a T7-like lytic phage for Pseudomonas fluorescens. BMC Biotechnology. BioMed Central **8**:80.

**Sulakvelidze, A., J. G. Morris Jr., Z. Alavidze, G. R. Pasternack, and T. C. Brown**. 2004. Method and device for sanitation using bacteriophages. U.S. Patent US 2004/0029250 A1.

**Thiel, K.** 2004. Old dogma, new tricks--21st Century phage therapy. Nature biotechnology **22**:31-6.

**Wagenaar, J. a, M. a P. Van Bergen, M. a Mueller, T. M. Wassenaar, and R. M. Carlton**. 2005. Phage therapy reduces Campylobacter jejuni colonization in broilers. Veterinary microbiology **109**:275-83.

**Wiedmann, M., D. Weilmeier, S. S. Dineen, R. Ralyea, and K. J. Boor**. 2000. Molecular and phenotypic characterization of Pseudomonas spp. isolated from milk. Applied and environmental microbiology **66**:2085-95.

**Yao, J. D. C., and R. C. Moellering Jr.** 1995. Antimicrobial agents, pp. 1474-1504. *In* P.R. Murray (ed.), Manual of Clinical Microbiology. American Society for Microbiology, Washington, D. C.

# 5. ANÁLISE TÉCNICA, COMERCIAL E DE IMPACTO AMBIENTAL E SOCIAL (EVTECIAS)

# Utilização de bacteriófago de *Pseudomonas fluorescens* no controle da gelificação do leite.

*Tecnologia, produtos e oportunidades de negócio*

Caracterização da tecnologia

A tecnologia proposta consiste na utilização de bacteriófagos no controle da atividade enzimática de bactérias.

Os bacteriófagos, chamados também de fagos, são vírus que infectam somente organismos procariontes, a exemplo das bactérias, possuindo ação bastante específica.

Estes micro-organismos são encontrados em números expressivos em seus habitats naturais, como água salgada, água doce, solo, material vegetal, corpos humanos e de animais (pele, cavidade oral, saliva, fezes) e até mesmo em alimentos[1].

Além da especificidade de sua ação, outra vantagem relevante da utilização de bacteriófagos está no fato de as bactérias possuírem dificuldade em obter resistência

[1]KENNEDY, J.E.; BITTON, G. Bacteriophages in foods. In: Goyal SM, Gerba CP, Bitton G (eds) Phage
ecology. Wiley, New York, 1987

a estes uma vez que os fagos se adaptam juntamente com elas, o que representa uma vantagem frente ao uso de antibióticos convencionais[2].

O uso de bacteriófagos vem aumentando em várias partes do mundo e seus campos de aplicação compreendem desde a manutenção da segurança da água e alimentos ao emprego na agricultura e saúde animal, conforme exemplifica a Figura 5-1[3].

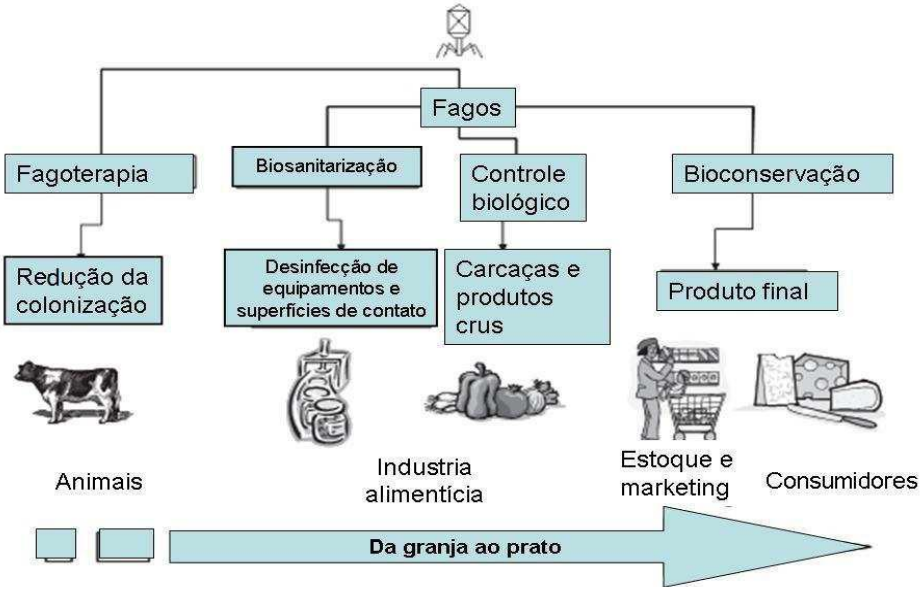



Figura 5-1. Campos de Aplicação - Bacteriófagos

Fonte: Instituto de Ciências Biológicas (ICB-UFMG)[4]

[2] Disponível em: <http://naturlink.sapo.pt/Natureza-e-Ambiente/Interessante/content/Bacteriofagos-como-alternativa-a-antibioticos-esquecidos-ou-simplesmente-ineficazes?bl=1&viewall=true>.Acesso em: 28 ago. 2012.

[3] Disponível em: <http://periodicos.ses.sp.bvs.br/scielo.php?script=sci_arttext&pid=S0073-98552010000200001&lng=es&nrm=iso> Acesso em: 29 ago. 2012.

[4] Disponível em: <http://microbiologia.icb.ufmg.br/monografias/157.PDF> Acesso em: 28 ago. 2012.

Oportunidade de aplicação da tecnologia

A partir da tecnologia descrita acima vislumbra-se a obtenção de uma solução fago-estável (solução estável contendo bacteriófagos) que será utilizada no controle da atuação enzimática da bactéria causadora da gelificação do leite, a *Pseudomonas fluorescens*.

A gelificação do leite é um processo deteriorativo ocasionado pela quebra de gorduras e proteínas por meio de proteases (enzimas que degradam proteínas) secretadas pela bactéria citada, ocasionando coagulação parcial e/ou aumento de viscosidade deste[5]. Vale ressaltar que estas enzimas são termorresistentes, portanto, permanecem neste alimento mesmo após o seu tratamento a altas temperaturas[6].

A bactéria causadora deste processo é pertencente à classe de micro-organismos psicrotróficos, ou seja, é capaz de se multiplicar em baixas temperaturas (≤ 7°C) [7].

Sob o ponto de vista da qualidade do leite, esta espécie bacteriana é a que mais contribui para a deterioração das propriedades organolépticas deste

---

[5] Disponível em: <www.agripoint.com.br/imagens/banco/MilkPoint/MilkPoint_Radar_Qualidade.pdf> Acesso em: 29 ago. 2012.

[6] Disponível em: <http://www.dominiopublico.gov.br/pesquisa/DetalheObraForm.do?select_action=&co_obra=145299> Acesso em: 19 set. 2012

[7] Disponível em: <http://www.zoonews.com.br/noticias2/noticia.php?idnoticia=176316> Acesso em: 28 ago. 2012.

alimento[8]. Alguns dos efeitos causados pela ação destas, além do anteriormente abordado, podem ser vistos na tabela abaixo.

Tabela 5-1. Efeito do crescimento de psicrotróficos no leite cru antes do tratamento térmico sobre a qualidade dos produtos lácteos

| **Produto** | **Efeito sobre a qualidade** |
|---|---|
| Leite UAT | Gelificação após 20 semanas.<br>Gelificação após 2-10 semanas, desenvolvimento gradual de sabores de sujo, amargo e envelhecido. |
| Leite em Pó | Redução da estabilidade térmica e aumento da capacidade de formar espuma em leite reconstituído. |
| Leite pasteurizado | Sabor de qualidade inferior quando comparado com o leite pasteurizado produzido com leite fresco. |
| Queijos duros | Rancidez.<br>Alteração no sabor, principalmente rancidez. Redução do rendimento de fabricação. |
| Cottage cheese | Correlação significante entre contagem de psicrotróficos no leite cru e sabor amargo. |
| Manteiga | Desenvolvimento mais rápido de rancidez feita a partir de leite refrigerado do que de leite fresco, lípase de *Pseudomonas* estava ativa na manteiga congelada. |
| Iogurte | Gosto amargo, sabor "sujo" ou de fruta, dependendo da microbiota presente. |

Fonte: elaborada pela equipe SIMI/UFV, adaptado de Cerqueira e Paiva[9].

[8] Disponível em:<http://www.agencia.cnptia.embrapa.br/Agencia8/AG01/arvore/AG01_182_21720039246.html> Acesso em: 29 ago. 2012.

[9] Disponível em: <http://multimedia.3m.com/mws/mediawebserver?mwsId=SSSSSufSevTsZxtUOYt9Px_UevUqevTSevTSevTSeSSSSSS--&fn=Impacto%20Qualidade%20MP.pdf> Acesso em: 29 ago. 2012.

Com o propósito de combater a gelificação, a solução contendo os fagos será adicionada ao leite cru contido nos tanques de resfriamento das propriedades rurais logo após a ordenha.

A adição do produto é dado neste momento de forma a otimizar a sua ação, uma vez que o acondicionamento deste alimento nestes recipientes desencadeia o desenvolvimento da bactéria a ser combatida[10].

Foi observado em testes laboratoriais que o bacteriófago utilizado neste projeto é capaz de inibir a quebra de proteínas do leite sem reduzir a população de sua bactéria hospedeira. Logo, levanta-se a hipótese de que o fago atue impedindo que estes micro-organismos secretem suas enzimas no leite, impedindo que ocorra a gelificação.

Matriz da gestão de plataforma

A *Pseudomonas fluorescens* está presente em diversos alimentos que são submetidos à refrigeração[11], devido a sua capacidade de crescer em baixas temperaturas. No entanto, a sua ação é mais expressivano leite,no qual é responsável por diversos efeitos negativos, conforme demonstrado na Tabela 5-1. Portanto, o foco dos pesquisadores é atuar dentro do setor produtivo leiteiro, conforme apresentado na Figura 5-2.

[10] Disponível em: <http://www.scielo.br/scielo.php?pid=S0103-84782006000200032&script=sci_arttext> Acesso em: 11 set. 2012.

[11] Disponível em: <http://www.ufpel.edu.br/faem/dcta/micro/pesquisa.htm> Acesso em: 06 set. 2012.

Para tal, os esforços da equipe são voltados para o desenvolvimento de uma solução fago-estável que, juntamente com a adoção constante de boas práticas de produção e manejo por parte dos produtores rurais e estabelecimentos processadores, atuará no biocontrole da bactéria *P. fluorescens*.

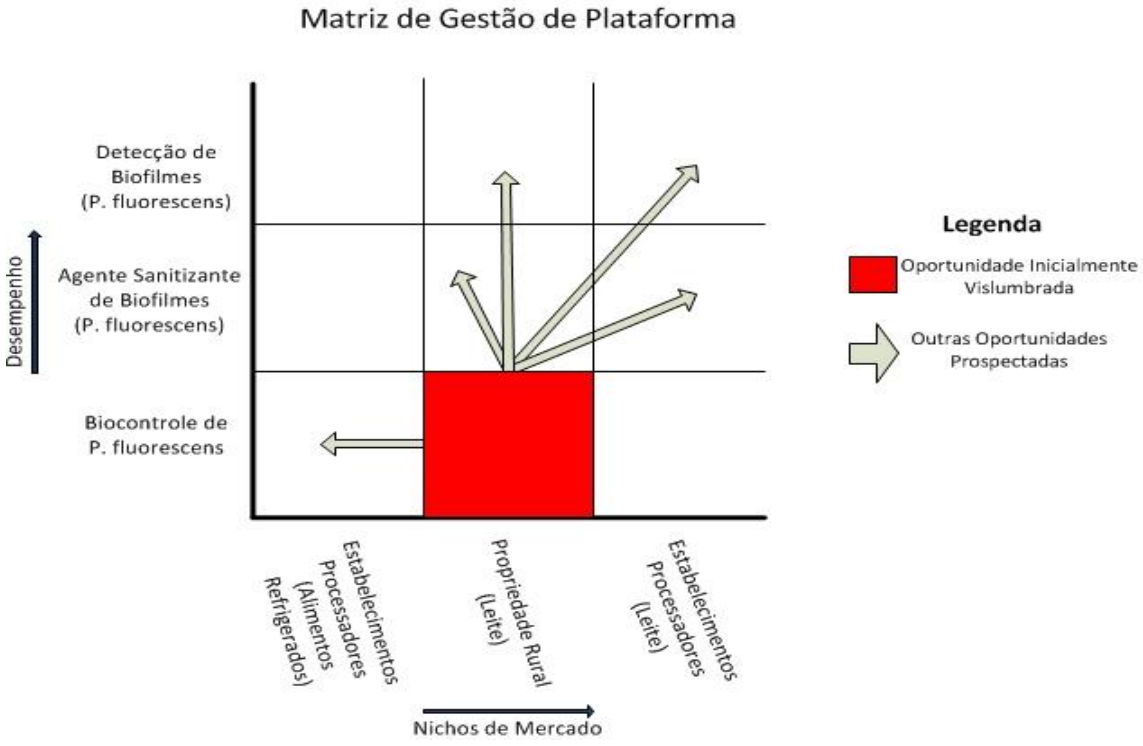



Figura 5-2. Matriz de Gestão de Plataforma

Fonte: elaborada pela equipe SIMI/UFV

A matriz de gestão de plataforma para a aplicação da tecnologia apresenta outras oportunidades que vão além da inicialmente pretendida pelos pesquisadores.

O bacteriófago em questão pode ser empregado como agente sanitizante onde atuará na prevenção da formação e na desinfecção de biofilmes consolidados de *Pseudomonas fluorescens* presentes em maquinários que entram em contato com o leite.

Biofilmes, por sua vez, podem ser definidos como um conjunto de bactérias aderidas a uma superfície. Esta organização consiste em uma forma séssil e adaptativa do ciclo biológico destes micro-organismos, com características distintas das formas bacterianas livres e circulantes[12].

O fago pode ser utilizado também como agente detector de biofilmes de *P. fluorescens*. A presença destes na superfície investigada será constatada através da emissão de fluorescência que ocorrerá quando houver a infecção deste conjunto de bactérias pelo micro-organismo utilizado em questão.

Além disto, o bacteriófago pode atuar no biocontrole de *P. fluorescens* em outras matrizes alimentares refrigeradas, onde o seu mecanismo de ação seria semelhante ao exercido em sua aplicação no leite.

Sistema de valor

A tecnologia em questão será empregada a fim de proporcionar maior qualidade ao leite através da manutenção de suas propriedades organolépticas, garantindo assim maior vida de prateleira para este alimento.

[12]Disponível em: <http://www.moreirajr.com.br/revistas.asp?fase=r003&id_materia=3934> Acesso em: 01 out. 2012.

Para tal, a inserção desta ocorrerá entre a produção e o processamento do leite, mais especificamente nos tanques de resfriamento presentes nas médias e grandes propriedades produtoras de leite, conforme indica a Figura 5-3. Seu uso refletirá nas etapas posteriores.

Os *players* diretamente envolvidos são os produtores de leite de médio e grande porte. Indiretamente temos os estabelecimentos processadores e o consumidor final.

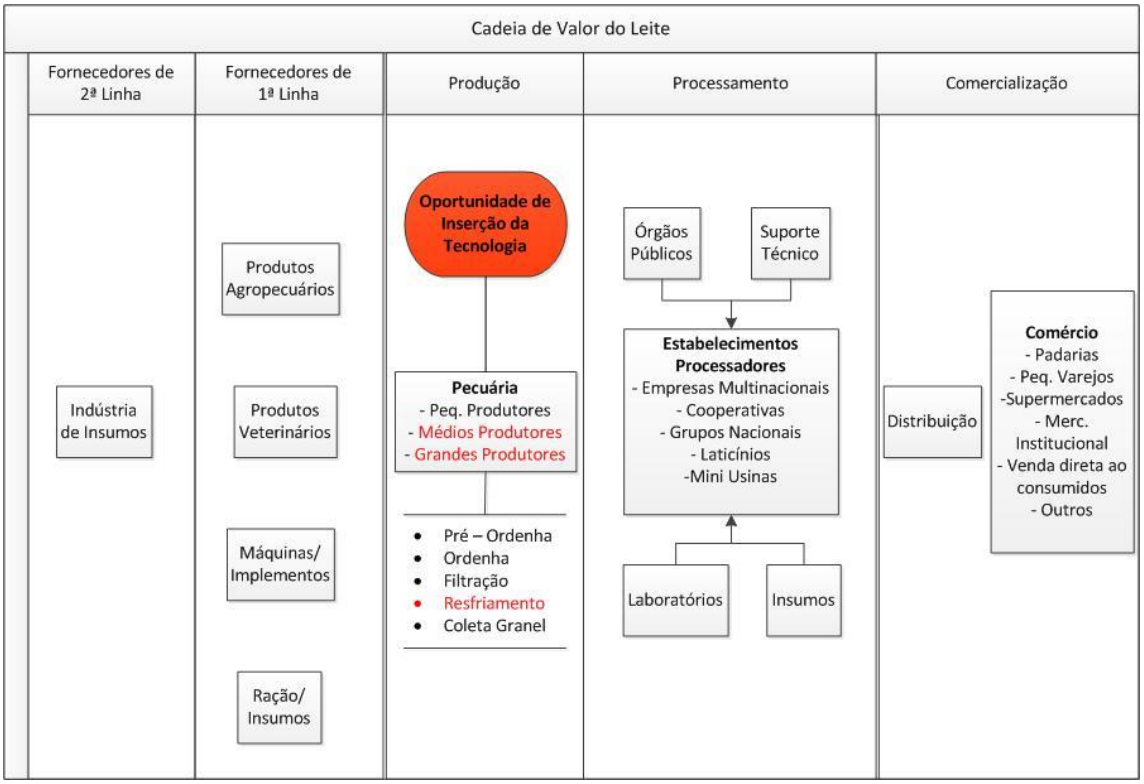



Figura 5-3. Cadeia de Valor do Leite

Fonte: elaborada pela equipe SIMI/UFV

*Aspectos regulatórios*

O produto que se pretende obter com a tecnologia deste estudo é considerado um aditivo alimentar. Segundo definição da Agência Nacional de Vigilância Sanitária (Anvisa), um aditivo alimentar é todo e qualquer nutriente adicionado intencionalmente aos alimentos sem o propósito de nutrir e sim com o objetivo de modificar as características físicas, químicas, biológicas ou sensoriais durante alguma etapa de produção do alimento[13]. Desta forma, seu uso deve ser avalizado por esta agência.

As Resoluções nº 45[14] e nº 46[15], publicadas pela Anvisa em 03 de novembro de 2010,tratam dos aditivos alimentares autorizados para o uso segundo as Boas Práticas de Fabricação (BPF). Estas resoluções não prevêem o uso de bacteriófagos conforme pretendido pelos pesquisadores, portanto a utilização destes fica submetida à obtenção prévia das autorizações/certificações necessárias. Este fato impacta negativamente o desenvolvimento do projeto.

O Ministério da Agricultura, Pecuária e Abastecimento (Mapa) publicou em 29 de Dezembro de 2011 a Instrução Normativa nº62 (IN62)[16], que estabelece que a temperatura máxima de conservação do leite nas propriedades rurais e em tanques comunitários é de 7°C e nas dependências do

[13] Disponível em: <http://www.milknet.com.br/?pg=informativo_ler&id=56&buscador=O-USO-DE-ADITIVOS-NA-INDUSTRIA-ALIMENTIC...&local=1> Acesso em: 11 set. 2012.
[14] Disponível em: <http://portal.anvisa.gov.br/wps/wcm/connect/11707300474597459fc3df3fbc4c6735/Resolu%C3%A7%C3%A3o+da+Diretoria+Colegiada++RDC+n++45+de+03+de+novembro+de+2010.pdf?MOD=AJPERES> Acesso em: 11 set. 2012.
[15] Disponível em: <http://www.diariodasleis.com.br/busca/exibelink.php?numlink=215515> Acesso em: 11 set. 2012.
[16] Disponível em: <http://www.sindilat.com.br/gomanager/arquivos/IN62_2011(2).pdf> Acesso em: 27 ago. 2012.

estabelecimento processador, 10°C. Esta faixa de temperatura é favorável ao crescimento da *Pseudomonas fluorescens*, indicando que o uso da tecnologia deste estudo promoveria o aumento da qualidade do leite mantido sob tais condições.

## *Mercado*

### Contexto de mercado e suas tendências

O mercado de leite brasileiro deve continuar crescendo em 2012. Tal crescimento será alavancado pelo fortalecimento da demanda doméstica que é projetada em aproximadamente 170 litros por habitante em 2012, um aumento de cerca de 2% em relação a 2011, porém ainda abaixo do recomendado pelo Ministério da Saúde, de 200 litros per capita por ano. O incremento das exportações, que segundo expectativa da associação Leite Brasil deve alcançar um patamar de 362 milhões de litros, alta de 15% em relação a 2011, também contribuirá para tal.

Após o volume de leite produzido em 2011 ter fechado com um crescimento de 1% em relação ao ano de 2010, número esse justificado pelos altos custos de produção que se abateu sobre os produtores, o montante

estimado para 2012 deve subir para 32,3 bilhões de litros, um avanço de 4%[17] conforme indicado na Figura 5-4.

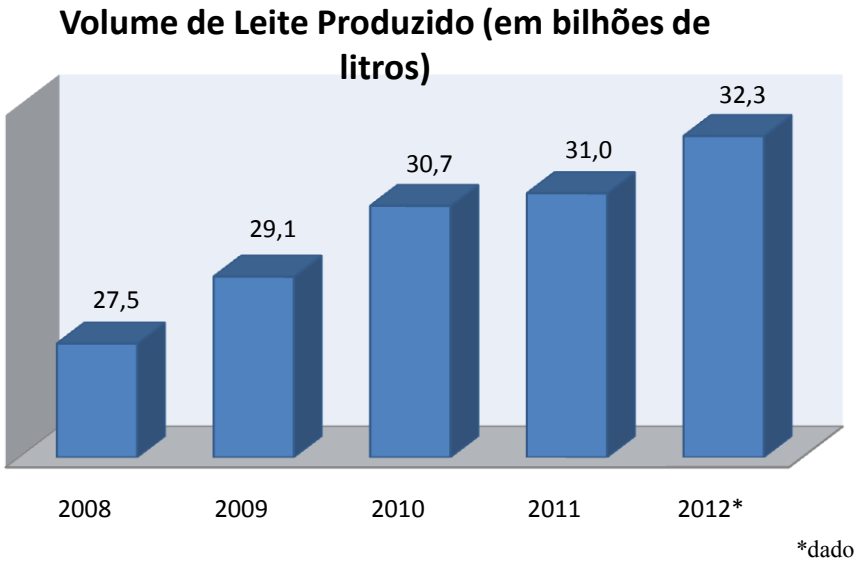



Figura 5-4. Produção de Leite no Brasil

Fonte: elaborada pela equipe SIMI-UFV, adaptado de Embrapa[18] e Revista Globo Rural[19].

O potencial produtivo do setor leiteiro e as vantagens comparativas do Brasil em relação a outras nações produtoras e tradicionalmente exportadoras são grandes. Isto deve-se ao fato deste país possuir condições climáticas favoráveis para a atividade, permitindo o pastejo dos animais na maior parte

[17] Disponível em: <http://revistagloborural.globo.com/Revista/Common/0,,EMI294991-18077,00-PRODUCAO+DE+LEITE+DEVE+CRESCER+EM.html> Acesso em: 29 ago. 2012.

[18] Disponível em: <http://www.cnpgl.embrapa.br/nova/informacoes/estatisticas/producao/tabela0230.php> Acesso em: 29 ago. 2012.

[19] Disponível em:<http://revistagloborural.globo.com/Revista/Common/0,,EMI294991-18077,00-PRODUCAO+DE+LEITE+DEVE+CRESCER+EM.html> Acesso em: 29 ago. 2012.

do ano, diminuindo assim os custos de alimentação, mão-de-obra e de capital empregado[20].

Outra tendência que influencia o setor produtivo é o crescimento e consolidação do pagamento por volume, regularidade e qualidade, promovendo escala e, sobretudo, a profissionalização da produção primária. A qualidade e higiene será uma preocupação constante, principalmente pela conscientização dos direitos do consumidor[21].

Dentre as 230 indústrias que analisam o leite no laboratório Clínica do Leite, 11 possuem programas de pagamento por qualidade. Dados da mesma instituição informam que as empresas que possuem essa medida verificam em seus fornecedores CBT média de 90.000 UFC/mL, contra 500.000 UFC/mL das que não o fazem[22].

Os incentivos ou bonificações variam entre as indústrias/cooperativas, mas de uma forma geral, para tal pagamento diferenciado observam-se fatores como: teor de gordura, teor de proteína, características sensoriais, além do

[20] Disponível em: <http://www.agencia.cnptia.embrapa.br/Agencia8/AG01/arvore/AG01_486_217200392422.html> Acesso em: 05 set. 2012

[21] Disponível em: <http://www.agencia.cnptia.embrapa.br/Agencia8/AG01/arvore/AG01_486_217200392422.html> Acesso em: 30 ago. 2012.

[22] Disponível em: <http://www.milkpoint.com.br/cadeia-do-leite/editorial/melhoria-da-qualidade-do-leite-reflexoes-41932n.aspx> Acesso em: 27 ago. 2012.

padrão microbiológico do leite que é obtido segundo taxas como a contagem de células somáticas (CCS) e contagem de bactérias totais (CBT) [23].

Dimensionamento de mercado

1) Estima-se que a produção de leite no Brasil atingirá 32,3 bilhões de litros no ano de 2012[24];

2) Do volume total produzido, tem-se que 81% são oriundos de produtores de médio e grande porte[25]:

*0,81 x 32,3 = 26,5 bilhões de litros de leite, aproximadamente.*

3) Tem-se que aproximadamente 30% do leite produzido no Brasil não são inspecionados[26], ou seja, não há garantias quanto a sua observação dentro de padrões estabelecidos por lei (refrigeração, teores de gordura e proteínas, padrões microbiológicos, dentre outros) uma vez que não passam pelo controle da indústria e do Mapa. Este leite tem destinos como o mercado informal, consumo interno da fazenda (familiares e animais).

---

[23]Disponível em: <http://www.milkpoint.com.br/cadeia-do-leite/conjuntura-de-mercado/pagamento-por-qualidade-situacao-atual-e-perspectivas-para-o-setor-lacteo-brasileiro-8211-parte-01-8089n.aspx> Acesso em: 31 ago. 2008.

[24] Disponível em: <http://revistagloborural.globo.com/Revista/Common/0,,EMI294991-18077,00-PRODUCAO+DE+LEITE+DEVE+CRESCER+EM.html> Acesso em: 03 set. 2012.

[25] Disponível em: <http://www.douradosagora.com.br/noticias/economia/grandes-e-medios-produtores-de-leite-detem-81-do-setor> Acesso em: 03 set. 2012.

[26] Disponível em: <http://www.cnpgl.embrapa.br/nova/informacoes/estatisticas/producao/tabela0231.php> Acesso em: 04 set. 2012.

*26,5 x 0,7 = 18,55 bilhões de litros de leite, aproximadamente.*

A inferência adotada neste cálculo é conservadora uma vez que o leite não inspecionado vem geralmente do pequeno produtor[27].

4) Segundo pesquisadores, é necessária a adição de 10ml de solução contendo os fagos em um volume de 1000 litros de leite cru para alcançar os fins que se pretende. Logo, a partir desta proporção, tem-se que o mercado potencial é de aproximadamente 185.500 litros de solução por ano.

0,01L --- 1000L

X --- 18.550.000.000L

X = 185.500L

*Nota:* Ainda não se chegou a conclusão do volume que conterá cada unidade do produto que pretende-se comercializar.

Relações de forças do mercado

Após a inserção da tecnologia no mercado leiteiro, esta irá se relacionar com os diferentes *players* deste conforme indica a figura abaixo.

[27] Disponível em: <http://www.agora.uol.com.br/saopaulo/ult10103u871376.shtml> Acesso em: 04 set. 2012.

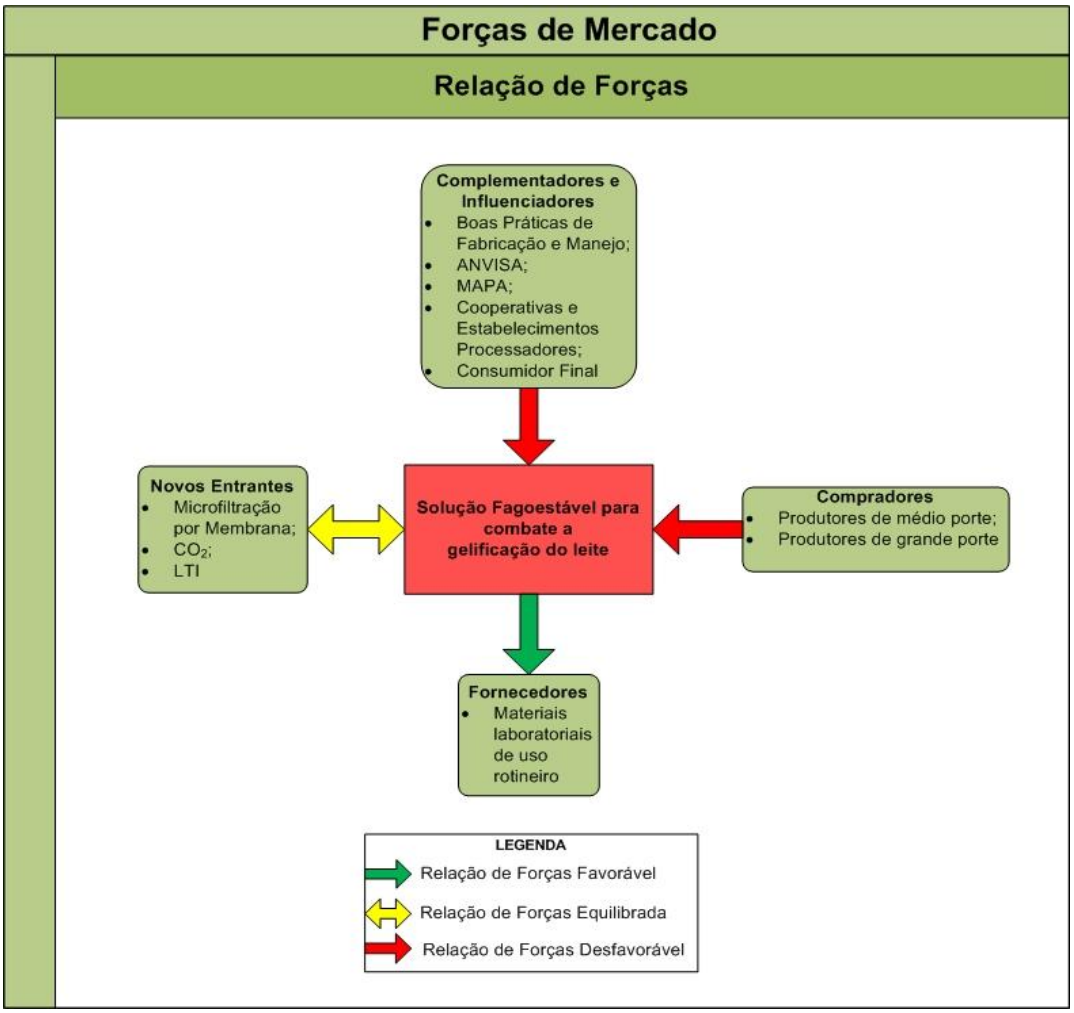



Figura 5-5. Forças de Mercado

Fonte: elaborada pela equipe SIMI-UFV

Fornecedores

A fim de se obter os bacteriófagos em maior quantidade para fins de comercialização, são necessários insumos tais como meios de cultura, reagentes químicos e materiais/equipamentos, todos estes de uso comuns e

rotineiros de laboratórios de pesquisa e, portanto, fornecidos por diversas empresas tais como *Sigma, Merck, Goldpharma e Himedia.* Logo, a obtenção destes ocorre de maneira facilitada.

Novos entrantes

Têm-se como novos entrantes da tecnologia proposta neste estudo os seguintes: processo de adição de $CO_2$ no leite de forma a controlar o crescimento de microrganismos psicrotróficas e, consequentemente, a sua ação[28]; processo de tratamento do leite por associação de altas e baixas temperaturas (LTI) de forma a desestabilizar as enzimas deterioradoras da bactéria *P. fluorescens*; processo de microfiltração por membrana do leite de visando a retenção de microrganismos indesejados[29]. Todas estas tecnologias citadas encontram-se em fase de estudos e suas respectivas inserções no mercado devem ser aprovadas pelos órgãos competentes.

Complementadores e influenciadores

A tecnologia deste estudo sofre influências dos seguintes:

A. *Órgãos governamentais*

---

[28] Disponível em: <http://www.milkpoint.com.br/radar-tecnico/qualidade-do-leite/adicao-de-cosub2sub-pode-aumentar-a-vida-de-prateleira-do-leite-pasteurizado-16154n.aspx> Acesso em: 20 set. 2012.

[29] Disponível em: <http://www.eufic.org/article/pt/tecnologia-alimentar/processamento-alimentar/artid/Filtracao-membrana-solucao-eficaz-melhorar-qualidade-alimentos/> Acesso em: 20 set. 2012

A ANVISA determina quais são os aditivos alimentares que possuem autorização para serem utilizados, logo, a comercialização do produto em questão deve ser primeiramente avalizada por esta agência.

O MAPA, por legislar sobre questões que abrangem toda a cadeia produtiva do leite,possui influência sobre a tecnologia objeto deste estudo.

B. *Cooperativas, estabelecimentos processadores e consumidores finais*

Tais *players* são influenciadores, uma vez que o consumidor final requer cada vez mais um produto de qualidade, conforme já abordado anteriormente.Para que esta demanda seja satisfeita, cooperativas e estabelecimentos processadores devem dispor de uma matéria prima (leite cru) de melhor procedência. Para tal, estes podem pressionar os produtores rurais quanto à utilização da solução fago-estável visando à melhoria da qualidade do leite.

Vale ressaltar que a adoção constante de boas práticas de manejo e fabricação por parte dos produtores atua como um complementador da ação da tecnologia frente à *Pseudomonas fluorescens* e os transtornos causados por esta.

*Equipe do projeto*

**Antônio Fernandes de Carvalho:** Técnico em Laticínios pelo Instituto de Laticínios Cândido Tostes, graduado em Farmácia e Bioquímica pela Universidade Federal de Juiz de Fora (UFJF), mestre em *Sciences Alimentares* pela *Universite de Caen*, doutor em *Sciences et Techiniques de Industries Agricoles* pela *Ecole Nationale Superieure Agronomique de Rennes (Ensar)* e

pós-doutor pelo *Institut National de la Recherche Agronomique*. Trabalhou por 8 anos como gerente industrial na área de laticínios e, durante este período, desenvolveu e registrou 73 produtos junto ao Mapa. Possui patente depositada na área de produtos lácteos funcionais. Atualmente é sócio proprietário do Laticínios Union LTDA e professor do Departamento de Tecnologia de Alimentos (DTA) situado na Universidade Federal de Viçosa (UFV).

**Maura Pinheiro Alves:** Graduada em Ciência e Tecnologia de Laticínios pela UFV, mestranda em Ciência e Tecnologia de Alimentos por essa mesma instituição. Possui experiência na área de alimentos, com ênfase em produtos lácteos.

**Monique Renon Eller:** Graduada em Bioquímica pela UFV, possui mestrado em Microbiologia Agrícola e desenvolve estudos em nível de doutoramento nesta mesma área com ênfase em Microbiologia Industrial e Biotecnologia, ambos pela mesma Universidade citada. Há 4 anos trabalha no isolamento e caracterização de bacteriófagos para uso industrial, em especial para o controle de contaminantes de alimentos.

**Roberto Sousa Dias:** Graduado em Bioquímica pela UFV, possui mestrado em Biologia Celular e Estrutural e desenvolve estudos em nível de doutoramento nesta mesma área, ambos pela mesma instituição citada. Possui experiência na área de virologia, com ênfase em bacteriófagos.

**Sérgio Oliveira de Paula:** Graduado em Medicina Veterinária pela UFV, mestre e doutor pelo Programa de Pós-Graduação em Imunologia Básica e Aplicada – Bioagentes Patogênicos pela Faculdade de Medicina de Ribeirão Preto (FMRP-USP). Possui experiência na área de fagoterapia

(utilização de bacteriófagos para controle de patógenos). Atualmente é professor do Departamento de Biologia Geral (DBG) situado na UFV.

*Estágio de desenvolvimento do projeto*

O projeto encontra-se na fase de caracterização do bacteriófago, conforme representa a Figura 5-6. As etapas necessárias ao desenvolvimento da tecnologia seguem esquematizadas abaixo de forma que as flechas mais escuras indicam um processo já concluído enquanto que as brancas referem-se a processos ainda não iniciados.


Figura 5-6. Estágio de Desenvolvimento do Projeto

Fonte: elaborada pela equipe SIMI-UFV

I. *Obtenção do Bacteriófago* (Abril/2011 – Setembro/2011)

Nesta fase os esforços dos pesquisadores se voltaram para a obtenção de um bacteriófago com alta capacidade de replicação e em quantidades e pureza suficientes para os testes seguintes.

Diante disto foram isolados 4 fagos e, após análise destes chegou-se a escolha de um que, por sua vez, apresentou resultados satisfatórios quanto a inibição da quebra de proteínas do leite.

II. *Caracterização* (Setembro/2011-Período atual)

Pretende-se nesta etapa aumentar o conhecimento acerca do fago de forma a facilitar a aprovação de seu uso junto aos órgãos competentes e a padronização de sua solução estabilizadora, além de mensurar a sinergia do bacteriófago com outros componentes como antibióticos, biocidas, dentre outros.

Uma parte especialmente importante deste processo de caracterização é a análise da gama de hospedeiros do fago. Este passo consiste na investigação acerca de quais são as bactérias que este microrganismo infecta.

Com os resultados de tal investigação é possível verificar se o bacteriófago ataca outras espécies de contaminantes indesejados além da *P. fluorescens*, a exemplo da *Escherichia coli*, bem como garantir que este não seja específico de bactérias lácticas que são importantes para o leite em seu processo de fermentação.

III. Testes de Atividade (2 anos)

Nesta etapa estão previstos testes acerca da ação do fago sobre biofilmes de *P. fluorescens* bem como a análise da produção de proteases (enzimas que degradam proteínas) por parte da bactéria hospedeira durante o seu processo de infecção pelo bacteriófago.

Estes também terão sua atividade avaliada quando submetidos a condições físico-químicas (pH, temperatura, salinidade, umidade, dentre outros) similares as do leite de forma a padronizar a solução que o manterá estável antes e após sua inserção neste alimento.

A partir dos resultados obtidos com estas ações pretende-se avaliar a viabilidade da utilização deste fago, verificar a necessidade de inserção de enzimas específicas em seu material genético, além de conhecer o seu modo de ação.

*IV. Certificações/Autorizações*

Esta fase será destinada a obtenção das autorizações/certificações necessárias para que a tecnologia deste estudo possa ser comercializada.

*Plano de desenvolvimento tecnológico*

Após a publicação da IN 51 através da qual regulamentou-se a respeito da obrigatoriedade da refrigeração do leite cru nas propriedades rurais, as pesquisas ganharam um apelo maior por buscar soluções para amortizar os transtornos causado por bactérias psicrotróficas, uma vez que estas possuem desenvolvimento favorecido nas temperaturas estabelecidas por esta Instrução.

Diante disto, a partir dos recursos (humanos, financeiros, P&D) descritos na Figura 5-7 abaixo, os pesquisadores envolvidos neste projeto estimam desenvolver uma solução fago-estável na qual detalhes quanto modo de ação e aplicação já foram abordados anteriormente neste estudo.

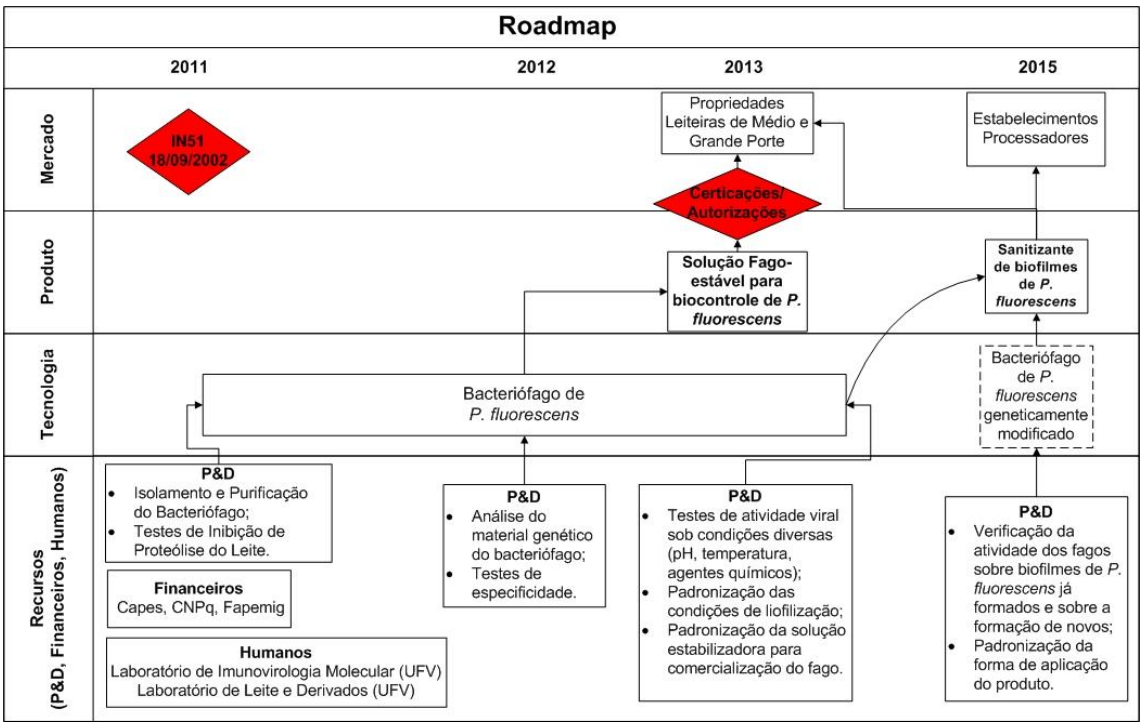



Figura 5-7. Plano de desenvolvimento tecnológico

Fonte: elaborada pela equipe SIMI-UFV

Espera-se ainda, como projeto de pesquisa em nível de pós-doutoramento da pesquisadora Monique Renon Eller, o desenvolvimento de um agente sanitizante de biofilmes de *P. fluorescens*.

Sua concepção se dará através da tecnologia da utilização do mesmo bacteriófago já isolado que, por sua vez, será testado quanto a sua ação sobre estes biofilmes. A partir dos resultados destes testes, irá se decidir quanto à necessidade da inserção de enzimas em seu material genético de forma a otimizar sua atividade. Estudos referentes à padronização da forma de aplicação deste produto também serão realizados.

Os recursos humanos e financeiros serão os mesmos empregados para o desenvolvimento da solução fago-estável.

*Barreiras e riscos relacionados ao projeto*

Em 2006, a *Food and Drug Administration* (FDA) aprovou a utilização de um pool de bacteriófagos denominado Listex P100 para o controle de *Listeria monocytogenes* em carnes e queijos[30].

Embora a utilização de bacteriófagos no setor produtivo de alimentos seja observada no cenário internacional conforme exemplo acima, no Brasil, legislações setoriais não prevêem o uso de produtos desenvolvidos a partir desta tecnologia em matrizes alimentares.

Diante disto, ações estão sendo tomadas a fim de se obter autorização legal para a utilização do produto que se vislumbra nesse projeto:

- Caracterização do fago quanto à sua classificação, morfologia, mecanismo de ação;
- Sequenciamento de seu material genético;
- Padronização de solução estabilizadora para comercialização do bacteriófago.

Com os resultados obtidos através destes esforços estima-se garantir a segurança da aplicação do bacteriófago em alimentos (a utilização destes não pode representar riscos para o ser humano) de modo a viabilizar a obtenção das autorizações/certificações necessárias para a sua comercialização.

[30] Disponível em: <http://periodicos.ses.sp.bvs.br/scielo.php?script=sci_arttext&pid=S0073-98552010000200001&lng=es&nrm=iso> Acesso em: 03 set. 2012.

Outro risco relacionado à tecnologia está em uma possível resistência da utilização desta por parte dos consumidores. A adição de partículas virais ao leite poderia ser considerada como um fator para a rejeição da compra do produto.

Porém, um marketing sobre os efeitos benéficos do uso de um agente biológico em detrimento do uso de reagentes químicos para o controle de contaminações poderia transformar essa limitação em uma vantagem estratégica.

*Impacto ambiental e relevância social*

A tecnologia ainda está em fase de desenvolvimento e até o momento não foram realizados estudos específicos acerca de seu impacto ambiental. No entanto, sabe-se que nenhum dos reagentes utilizados na produção da solução fago-estável apresenta risco biológico de contaminação.

*Conclusão sobre o projeto*

O produto vislumbrado tem por finalidade agregar qualidade ao leite via combate à ação da bactéria *Pseudomonas fluorescens*, causadora do processo de gelificação nesta matriz alimentar. Sua utilização possui vantagens, uma vez que os bacteriófagos não são organismos patógenos dos seres humanos e são inertes neste alimento, ou seja, sua adição não altera as propriedades organolépticas do leite.

Para a consolidação da tecnologia e sua posterior tentativa de inserção no mercado, os esforços em pesquisa e desenvolvimento (P&D) frente às etapas restantes do projeto são bem definidos e, os resultados obtidos até o momento são positivos.

A equipe envolvida neste trabalho, por sua vez, possui as competências técnicas necessárias para a conclusão deste, uma vez que é formada por uma mescla de profissionais com experiência na área de produtos lácteos e também no uso de bacteriófagos para controle de contaminantes em alimentos.

Além disso, os recursos financeiros e de infra-estrutura a que se tem disposição são igualmente satisfatórios.

# 6. GENERAL CONCLUSIONS AND PROSPECTS

In this study:

- ✓ The phage UFV-P2 was successfully isolated from waste water obtained from a dairy plant in Viçosa, Minas Gerais, and presented high capability to multiplying in the proteolytic strain of *Pseudomonas fluorescens* 07A previously isolated from milk;
- ✓ The phage was able to reduce α, β and κ-casein proteolysis in LDR 12% from 4.09, 6.08 and 0.01% of the initial amount, to 76.02, 100.00 and 28.80%, respectively, and to slow milk deterioration at room temperature, although it did not inhibit bacterial growth in this environment;
- ✓ The UFV-P2 virion particles have isometric capsids of 40 to 50 nm in diameter and very short tails, and can be classify as belonging to the *Podoviridae* family, order *Caudovirales*;
- ✓ The UFV-P2 genome was sequenced and analyzed *in silico*. It is a linear 45,517 bp DNA genome with a GC content of 51.5%, and 41 ORFs annotated to five different protein groups: i) hypothetical proteins; ii) scaffolding protein; iii) constitutive proteins; iv) structural proteins, and v) enzymes.
- ✓ Bioinformatics' analysis showed that UFV-P2 belongs to the LUZ24-like genus and possess high identity with the phages PaP3, MR299-2, LUZ24 and tf, phages specific to different *Pseudomonas* species and isolated in distinct countries.
- ✓ The phage possess at least five main structural proteins ranging from 15 to 60 kDa, including a major head and a tail fiber protein, which were theoretically associated to the annotated ORFs according to their molecular sizes.

Thus, the phage isolated in this work could be a strong candidate for the production of additives and sanitizers that could be use to prevent and control these contaminants, avoiding the loss of thousands of liters of milk. For this, studies will be conducted to evaluate its host range and its effectiveness in reducing biofilm formation and dispersing consortia of biofilms formed by the main bacteria present in the industrial environment. Further analysis should also disclose their mode of action, confirming or rejecting the hypothesis that UFV-P2 negatively regulates transcription of genes of proteases and other cellular enzymes.