TÉCNICO LISBOA

Genomic Characterization of Three Novel *Acidovorax* Phage Genera and Their Potential in Phage Biocontrol

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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October 2021

Preface

The work presented in this thesis was performed at the Laboratory of Gene Technology, Department of Biosystems of the Katholieke Universiteit Leuven (Leuven, Belgium), during the period February-July 2021, under the supervision of Dr. Jeroen Wagemans and Dominique Holtappels, and within the frame of the Erasmus programme. The thesis was co-supervised at Instituto Superior Técnico by Prof. Miguel Teixeira.

Declaration

I declare that this document is an original work of my own authorship and that it fulfills all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

Acknowledgments

This master's thesis would not have been possible to conclude without the support of colleagues, friends and family, so I would like to express a few words of gratitude.

First of all, I would like to thank all my supervisors for all the time and guidance dedicated to me. To Dr. Jeroen Wagemans, thank you for giving me the opportunity to work in LoGT. Also, for reading my thesis at an incredible speed and for all the remarks and suggestions you made. To Prof. Miguel Teixeira, thank you for the support through all this time. And finally, a huge thank you to my daily supervisor, Dominique Holtappels, for such an amazing guidance. Thank you for your availability and accessibility, for all the corrections and insights, and especially for all your energy and enthusiasm that turned every moment better. Thanks a lot for everything that you have taught me.

My thanks also go to all the team from the Laboratory of Gene Technology, who accompanied me through the whole semester and helped me everytime I needed. Thank you for making me feel welcome in the lab, for the warm cooperation and great atmosphere despite the Corona-measures.

A special thank you to all my family. To my parents and grandparents, for everything they did for me and for the unconditional support. To my sisters, that believed in me like no one else, always giving me confidence and that, despite the distance, never left me alone. A big and very special thank you to Elena, for all the help, time and patience, for always encouraging me to do better in such an honest and direct way, and for never letting me go down in moments of stress.

Abstract

Bacterial black spot, caused by *Acidovorax valerianellae*, is responsible for significant yield losses in lamb's lettuce (*Valerianella locusta*) in many producing countries, especially in Europe. To date, no resistant varieties of *V. locusta* are available that effectively control the disease under field conditions. Moreover, concerns over the environmental impact of chemical pesticides and the development of bacterial resistance to antibiotics has urged the discovery of new approaches for disease management. Bacteriophage-based biocontrol has been suggested as a sustainable and natural alternative strategy to combat bacterial pathogens. In this study, novel phages infecting *A. valerianellae* and *A. cattleyae*, Alfacinha1, Alfacinha3, Acica and Aval, were isolated and characterized, being representative of three new phage genera. Aval and Acica phages revealed genomic features characteristic of temperate lifestyle, encoding toxins likely associated with lysogenic conversion, which is in sharp contrast to Alfacinha1 and Alfacinha3 phages, that displayed a lytic lifestyle. Alfacinha3 was selected for application as a biocontrol agent during seed steeping. It could achieve an 87% reduction in bacterial concentration on artificial infested seeds, and an increase in germination rate from 58.9% to 93.3%. Additionally, after 22 days of growth, the infected seedlings had a dramatic reduction in vigor index, whilst the phage-treated ones had a vigor index similar to the negative control, reinforcing the ability of bacteriophages to effectively reduce disease progression. This study shows how genomic analyses represent an essential route to ensure safe phage application and demonstrates the potential of a phage-based biocontrol strategy against *A. valerianellae*.

Keywords: *Acidovorax*, Bacteriophages, Biocontrol, Lamb's lettuce, Genome analysis

Resumo

O patógeno *Acidovorax valerianellae* provoca uma doença caracterizada por manchas pretas nos canónigos (*Valerianella locusta*), responsável por perdas consideráveis no rendimento em muitos países produtores, especialmente na Europa. Atualmente, nenhuma variedade resistente de *V. locusta* foi encontrada e não é possível controlar eficazmente esta doença. Além disso, a preocupação com o impacto ambiental dos pesticidas e desenvolvimento de resistência aos antibióticos tem obrigado a procura de novas abordagens para o controlo de doenças. Nesse sentido, o biocontrolo baseado em bacteriófagos tem surgido como uma estratégia alternativa sustentável. Neste estudo, os primeiros fagos a infetar *A. valerianellae* e *A. cattleyae*, Alfacinha1, Alfacinha3, Acica e Aval, foram isolados e caracterizados, sendo representativos de três novos géneros. Os fagos Aval e Acica revelaram características genómicas particulares de atividade lisogénica, codificando toxinas associadas a conversão lisogénica, em contraste com os fagos Alfacinha1 e Alfacinha3, que mostraram seguir um ciclo de vida lítico. Alfacinha3 foi selecionado para tratamento de sementes contra *A. valerianellae*, permitindo atingir uma redução de 87% na concentração bacteriana dentro da semente e aumentar a taxa de germinação de 58,9% para 93,3%. Além disso, após 22 dias de crescimento, as plantas infetadas apresentaram uma redução acentuada no índice de vigor, ao contrário daquelas tratadas com o fago cujo índice de vigor era semelhante ao controlo negativo, reduzindo, portanto, a progressão da doença. Este estudo mostra como a análise genómica representa uma ferramenta essencial para garantir a aplicação segura de fagos e demonstra o potencial do biocontrolo baseado em fagos contra *A. valerianellae*.

Keywords: *Acidovorax*, Bacteriófagos, Biocontrolo, Canónigos, Análise genómica

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List of Acronyms

Abi	Abortive infection
ASM	Acibenzolar-S-methyl
BFB	Bacterial fruit blotch
BLAST	Basic Local Alignment Search Tool
Bp	Base pair
CFBP	Collection Francaise de Bactéries Phytopathogènes
CFU	Colony forming unit
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
DNA	Deoxyribonucleic acid
dsDNA/RNA	Double stranded DNA/RNA
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substances
FAO	Food and Agriculture Organization
GBBC	Culture collection of plant pathogenic bacteria at ILVO
HGT	Horizontal Gene Transfer
HTH	Helix-turn-helix
HTP	High-throughput
ICTV	International Committee on the Taxonomy of Viruses
ICNV	International Committee on Nomenclature of Viruses
ILVO	Instituut voor Landbouw en Visserijonderzoek
IPSP-CNR	Istituto per la Protezione Sostenibile delle Piante
kb	Kilobase(s)
LB	Lysogeny Broth
LB_{ms}	Lysogeny Broth, medium salt
LMG	Laboratory of Microbiology of Ghent University
LPS	Lipopolysaccharide
MOI	Multiplicity of infection
MS	Murashige and Skoog basal medium

MTase	Methyltransferase
OD	Optical density
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PCG	Provinciaal Proefcentrum voor de groenteteelt Oost-Vlaanderen
PEG	Polyethylene glycol
PFU	Plaque forming unit
PHB	Poly-3-hydroxybutyrate
PHBV	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer
PMVE	Polymethyl vinyl ether
PSKW	Proefstation voor de groenteteelt Sint-Katelijne Waver
pv	Pathovar
QS	Quorum sensing
RBP	Receptor binding protein
RHS	Rearrangement hotspot
RNA	Ribonucleic acid
RPM	Rotations per minute
SAR	Systemic acquired resistance
SDS-PAGE	Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
SmR	Streptomycin-resistance
ssDNA/RNA	Single stranded DNA/RNA
TEM	Transmission electron microscopy
USA	United States of America
UV	Ultraviolet

Chapter 1

Introduction

Each year, the world faces an increasing challenge regarding crop production. While a boost in the agricultural yield is crucial to satisfy the global growing demand, agriculture is, on other hand, limited by the increasing scarcity and diminishing quality of land and water resources. Moreover, climate change already negatively affects all agricultural sectors, and this impact is likely to become stronger in the coming years if no substantial investments are made to develop and implement more resource-saving and climate-friendly technologies. A changing course is thus critical to avoid a future characterized by persistent food insecurity and unsustainable economic growth. A sustainable way forward is to improve the performance and efficiency of the current croplands rather than continue to expand and clear more areas. For this purpose, major constraints, such as bacterial plant diseases, should be minimized. Given the emergent crisis of antibiotic-resistant pathogens and the lack of efficient methods to control them, along with the increasing demand for chemical-free food, there is a high demand for research in sustainable and natural pest control strategies. Bacteriophages are emerging as a suitable alternative for more consumer-friendly organic farming, since they are antibacterial agents that occur naturally in the environment, posing no harm to humans who are in constant exposure to them.

In recent years, phage biocontrol has indeed shown promising outcomes in a number of major bacterial plant diseases. However, despite the growing benefits of phage application in different crops, phage biocontrol studies in *Acidovorax* pathogens, responsible for heavy economic losses in a wide array of plants, have hardly been reported. One major target is lamb's lettuce, mainly sold as a ready-to-eat vegetable. This market has recently gained significant attention, given the increasing consumer interest in practical and labeled products, leading to higher prices and, in turn, higher economic benefits for those involved in the production chain.

The overall objective of this thesis was to characterize novel bacteriophages and evaluate their potential in the biocontrol of *Acidovorax valerianellae*, the causing agent of black spot disease in lamb's lettuce. As a first step, bacteriophages were isolated from soil samples, and their host range was screened using an established collection of diverse *Acidovorax* strains (Chapter 4.1). The phage isolates were subsequently characterized in depth to assess their suitability and safety for application in biocontrol (Chapters 4.2 and 4.3). This included a genomic characterization by whole genome sequencing to unravel basic genomic features and to prospect the diversity of host-phage interactions. As newly sequenced phages, the relationship with other known viruses was also explored based on genome-wide sequence similarities along with phylogenetic analyses. Chapter 4.2 focuses on the characterization of one of the isolated bacteriophages, the orphan phage Aval, and explores its infection route. Chapter 4.3 includes the characterization of the other clade of isolated phages, Alfacinha1, Alfacinha3 and Acica. In a last phase, based on the infection strategy, the best phage candidate, Alfacinha3, was selected for further microbiological assays (Chapter 4.3.3), including investigation of speed and efficiency of ad-

sorption and infection, and for a seed bioassay to assess its efficacy as seed-coating agent against *A. valerianellae* (Chapter 4.3.4).

In summary, this research project provides a proof of concept for the use of bacteriophages as a potential alternative to the current control techniques to combat bacterial plant diseases.

Chapter 2

Literature Review

2.1 Importance of Crop Protection

According to an estimate by the Food and Agriculture Organization (FAO), the world population will reach almost 10 billion by 2050 [1]. The role of agriculture in continuously providing safe food to a growing global human population is thus of utmost importance. Even more, sustainability is key to minimize the impact on the environment, given the threat of climate change, biodiversity loss and freshwater eutrophication [2]. Several predictions have shown that global crop production needs to double by 2050 to keep up with the projected demands from a rising population, diet transitions, and increasing biofuels consumption. Nevertheless, the world is now facing a major challenge given that the agricultural yields are not improving accordingly to meet that increase [3]. Even though, opportunities do exist to boost crop production, wherein the more efficient use of the current arable area has been highlighted as a preferred solution over clearing more land. The agricultural expansion of croplands has been prevented due to the high environmental cost to biodiversity and carbon emissions [4]. Some additional strategies should also be noted to reduce this expected demand growth in food, namely reducing food waste and modifying to a more plant-based diet [3].

In order to boost crop production, limiting factors need to be minimized. In this respect, bacterial plant diseases place major constraints on crop production and account for significant annual losses up to 40% on a global scale, according to the FAO, representing an important threat to the food security and economy [5]. However, the identification and deployment of disease management solutions for bacterial diseases still remain a formidable challenge.

2.1.1 Pest Management Strategies

Chemical bactericides, such as copper-based compounds and antibiotics have been the most common and effective way of controlling disease outbreaks in many pathosystems in the absence of permanent and robust host disease resistance. Although they have been relatively successful disease management tools, its extensive use over multiple years is correlated with the selection of resistance in pathogen populations [6].

Streptomycin has, for example, been the most widely used antibiotic for plant disease control since its introduction in 1955 [7]. It has been used primarily for the control of fire blight by *Erwinia amylovora*, but also targeting other pathogens such as *Pseudomonas syringae*, *Xanthomonas campestris* and *Agrobacterium tumefaciens* [8]. Due to its longest use for the treatment of a wide range of crops throughout the largest geographic region, streptomycin resistance is now quite widespread among plant-pathogenic bacteria. The majority of streptomycin-resistant (SmR) plant pathogens encode the transmissible SmR

transposon Tn5393, originally isolated from *E. amylovora*, that harbors *strAB*, a tandem resistance gene pair that confers streptomycin resistance through inactivation of the streptomycin molecule through either phosphorylation or adenylylation [8, 9].

Despite the use of antibiotics has generally been discouraged, a few others, such as oxytetracycline, gentamicin and kasugamycin, have been used to control various crop diseases caused by species including *Erwinia*, *Pectobacterium*, *Pseudomonas*, *Ralstonia*, and *Xanthomonas*, and especially in areas where streptomycin is no longer effective because of resistance [7, 10]. These antibiotics have, however, also been linked to incidences of resistance. For instance, tetracycline resistance was reported in a few plant-pathogenic bacteria, including *P. syringae* [11] and *A. tumefaciens* [12], and resistance to kasugamycin was reported for two bacterial rice pathogens, *Acidovorax avenae* and *Burkholderia glumae* [13]. Although there are few reports of resistance, multiple tetracycline resistance genes are present within the genomes of several different plant-pathogenic bacteria, including multiple *Xanthomonas* and *Pseudomonas* species, *Ralstonia solanacearum* and *Erwinia piriflorinigrans* [14]. Given the horizontal gene transfer and the acquisition of resistance genes by plant pathogens in many ecosystems, it is to be expected that with any newly introduced antibiotic there will be an evolution of resistance.

As a result of the recent restrictions on general antibiotics and chemicals to ensure public health and to limit the occurrence of resistant strains, the search for sustainable, natural biocontrol of bacterial pathogens has reached a critical stage, especially given the increased food production demand [15]. Governments, such as the European Commission, agreed to establish integrated pest management strategies as the standard for crop protection. These strategies are based on the implementation of sustainable pest control strategies with the emphasis on biological control not to eradicate pests, but to maintain their populations to avoid economical losses [15]. An integrated management approach can include the use of plant host resistance or the growth of less susceptible cultivars, cultural practices directed at inoculum reduction, but also the intervention with chemical and/or biological controls. The latter can be broadly defined as the use of beneficial microbes or their byproducts or byproducts/extracts from plants or animals in the suppression of plant disease [6]. To develop such strategies, a thorough understanding of the ecology of potential antagonists, interactions with plants and with its microbiome and interactions with pathogens is needed for improved and sustainable deployment in commercial systems. Functional genomic analyses appear to represent a solid route to obtain knowledge on current gold-standard biocontrol agents [6].

A questionnaire launched by Mansfield and colleagues in 2012, asking for the most prominent bacterial species in plant pathology includes *P. syringae*, *A. tumefaciens* and *E. amylovora* [16]. Nevertheless, *Acidovorax* spp. infecting cucurbits, cereal crops, lettuce, among others, are also prominent bacterial species causing losses in the respective crops. In this literature review, we will dig deeper into this bacterial genus and discuss the implementation of phage biocontrol to control the diseases caused by these bacterial species.

2.2 *Acidovorax* Plant Diseases

2.2.1 General description of *Acidovorax* genus

The genus *Acidovorax* was established in 1990 and initially contained only the non-phytopatogenic species *A. facilis*, *A. temperans* and *A. delafeldii* [17]. The plant pathogenic members of the genus, originally classified in the genus *Pseudomonas*, were transferred into the genus *Acidovorax* in 1992 by Willems *et al.* [18]. The genus *Acidovorax* belongs to the beta division of the Proteobacteria [17] and includes a variety of species that exhibit distinctive lifestyles. From the *Acidovorax* described species, it is shown that some are well adapted to water and soil environments while others can interact with

eukaryotic organisms, acting mainly as phytopathogens. Among the latter, some species were found to cause disease to a variety of agriculturally and economically important crops, including *A. citrulli*, *A. avenae*, *A. oryzae*, *A. cattleyae*, *A. konjaci*, *A. anthurii*, and *A. valerianellae* [18, 19, 20, 21], that are summarized in Table 2.1. On the other hand, some of the *Acidovorax* environmental species occupying water and soil habitats have shown interesting features due to their ability to degrade environmental pollutants, including arsenic removal from wastewater [22], capability of degrading human-made toxic compounds polychlorinated biphenyl/biphenyl and 2-nitrotoluene [23, 24] and biodegradation of some commercial polyesters such as PHB and PHBV [25].

Table 2.1: Plant pathogenic members of the *Acidovorax* genus.

Pathogen	Host	Disease
A. anthurii	*Anthurium spp.*	Leaf-spot
A. avenae	*Poaceae*	Red stripe of sugarcane; Leaf blight of maize and sorghum
A. cattleyae	*Cattleya spp.*, *Phalaenopsis spp.*	Leaf-spot
A. citrulli	*Cucurbitaceae*	Fruit blotch
A. konjaci	*Amorphophallus konjac*	Leaf-spot
A. oryzae	*Oryzae sativa*	Brown stripe
A. valerianellae	*Valerianella locusta*	Leaf-spot
Acidovorax sp.	Geranium and petunia	Leaf-spot

2.2.2 *Acidovorax* spp. are notorious pathogens infecting a wide array of different plant hosts

As summarized by Table 2.1, *Acidovorax* consists of diverse species of bacteria infecting a wide array of plants. One of the most prominent species in this genus is *A. avenae*. This species was transferred from the genus *Pseudomonas* to the genus *Acidovorax* by Willems *et al.* (1992) [18] as three subspecies: *A. avenae* subsp. *cattleyae*, *A. avenae* subsp. *citrulli*, and *A. avenae* subsp. *avenae*. However, a study was performed by Schaad *et al.* (2008) [21] to re-evaluate the phylogenetic relatedness among the above phytopathogenic *A. avenae* subspecies. Genetic (16S rDNA and 16S–23S rDNA internal transcribed spacer (ITS) region sequencing, DNA/DNA reassociation assays, AFLP analysis) and phenotypic data, including fatty acid profiles, revealed four distinct genotypes among the *A. avenae* strains, supporting an emendation of the species. Therefore, nowadays, several authors adopt the reclassification up to species level as *A. avenae*, *A. cattleyae*, *A. citrulli*, and the new species *A. oryzae* for the rice isolates. *A. avenae* infects *Poaceae* family members, including maize, sorghum, corn, oat, barley, rye, various millet strains, vasey grass, and sugarcane [26, 27]. Recently, *A. avenae* causing bacterial leaf blight on tea was also demonstrated [28]. On sugarcane, *A. avenae* causes red stripe and top rot [29]. This disease affects sugarcane crops practically worldwide, being present in more than 50 countries, and is related to serious global losses in yield [26, 30, 31]. Symptoms appear on the leaves and leaf sheaths as water-soaked stripes that gradually turn reddish and a stem rot which normally begins near the growing point. It can significantly reduce theoretically recoverable sucrose when its incidence exceeds 25% [32]. Also, severe infection of sorghum has been reported to occur under greenhouse conditions. The leaves of sorghum infected by the pathogen develop a greyish-green color with red borders or small, irregular and reddish necrotic stripes which may coalesce to form extensive necrotic areas covering a large proportion of leaf [29]. Despite its importance, there is little information on the use of chemicals to control diseases caused by *A. avenae*. However, copper sulphate

and streptocycline (which is a combination of streptomycin and tetracycline) have been used to control infection in maize [29].

Another economical relevant pathogen within the *Acidovorax* species, *A. cattleyae* was first isolated from diseased orchids with leaf spot by Pavarino (1911) in Italy [33], as *Pseudomonas cattleyae*. Infections by this bacterium in *Cattleya spp.*, *Phalaenopsis spp.* and their hybrids have been reported in Australia, Italy, the Netherlands, Philippines, Portugal, Taiwan, Poland and the USA [34, 35, 36], resulting in heavy losses to the orchid industry. The pathogen enters the plant through the stomata [37], and symptoms begin as small, dark green, water-soaked spots on the bottom of the leaves. These lesions increase in size rapidly to kill the entire leaves and possibly even invading the growing point of the plant, causing death [38]. Usually, the color turns from light to dark brown with age and older spots may be surrounded by a light green or yellow halo. Despite its relevance to the horticultural sector and even though this disease has been detected often over the last years very little research has been published about it.

This is in sharp contrast to *A. citrulli*, which has been the focus of several studies in literature. This member of the *Acidovorax* genus causes bacterial fruit blotch (BFB), an economically important disease in the cucurbit production industry. The pathogen was first reported by Webb and Goth (1965) [39] as the causal agent of BFB in commercial watermelon fields in Georgia. Since then, it was also reported in several other countries around the world, representing nowadays a serious threat to cucurbit crop production worldwide [40, 41, 42]. Although the pathogen is known to mainly infect watermelon and melon, BFB has been reported in many other cucurbits such as pumpkin, honeydew, cucumber, squash, anguria fruit, burr gherkin and gramma (*Cucurbita moschata*) [29, 41]. Apart from cucurbitaceous species, *A. citrulli* was also isolated from diseased eggplant seedlings and tomato seeds [43]. Symptoms of BFB can appear on all developmental stages of plant growth and include water-soaked lesions on cotyledons, hypocotyls, and leaves, and also light brown-reddish colored lesions on the leaves. In addition, small and irregular water-soaked regions on a fruit surface progress through the rind, resulting in decays and cracks, that can lead to total fruit loss, resulting in large economic losses up to 90% [44]. The application of copper-containing bactericides or antibiotics on infested fields showed to be ineffective [45], and to date there is still no BFB-resistant cultivars commercially available [40]. It is reported that *A. citrulli* displays remarkable longevity in stored cucurbit seeds [41, 46], and since it is a seed-borne disease, the use of pathogen-free seed has been a priority for disease control.

A. oryzae (formerly *A. avenae* subsp. *avenae*) that has been gaining attention more recently, is the causal agent of bacterial brown stripe of rice (*Oryza sativa*). This disease has caused heavy economic loss in many countries in the whole world, with special incidence in rice-growing regions of East Asia; China, South Korea and Japan [47, 48]. The contaminated seeds represent the main source of inocula for outbreak of the disease [49]. The first symptoms appear as brown stripes on the lower part of the leaf sheath and frequently extend into the sheaths by spreading along the leaf midrib [50]. Rice is expected to be a leading crop to feed rising world's population, which is expected to increase by 70% in 2050 [51, 52]. Therefore, it is of great importance to find an adequate and sustainable control strategy. Several chemical antimicrobial agents in the form of pesticides and antibiotics (triazoles, tetracycline, nalidixic acid, chloramphenicol and sulfamethoxazole) have been used to control and prevent rice diseases [53, 52]. However, it is not only expensive and environmentally corrosive but also leads to pathogen resistance against these chemicals. Recently, nanotechnology has emerged as an alternative to conventional chemical control methods. Several studies have reported the use of metal nanoparticles for its potential antimicrobial activity against the rice pathogens such as *A. oryzae* [52, 54, 55, 56, 48, 57].

Some other plants have also been the target of diseases caused by *Acidovorax* pathogens. For instance, *A. anthurii* causes bacterial leaf spot of anthurium (*Anthurium andreanum*), an important ornamental cash crop found in most tropical-humid countries, particularly the Caribbean, Latin America

and Hawaii. It has been identified as a serious limiting factor for commercial anthurium production, especially in the French West Indies and Trinidad and Tobago [19]. The pathogen enters the vascular system of anthurium leaves via natural openings or wounds. The disease begins as necrotic lesions close to the veins and leaf margins which can rapidly become in large, black necrotic spots which turn grey on older leaves. Infection can become systemic resulting in tissue discoloration and lead to eventual plant death [58]. It is still a challenge to control this disease since cultural control methods have proven to be inadequate in controlling the disease, related with the phytotoxicity of copper for *Anthurium spp.*, and due to the lack of resistant varieties [58, 59]. It is mostly argued that the development of resistant cultivars is the only means of sustainable management of this disease [59], and there is currently some effort to investigate the genetics of resistance and improve breeding efficiency in order to revitalize the anthurium industry [60].

Another example is *A. konjaci*, previously described as *Pseudomonas pseudoalcaligenes* subsp. *konjaci* [61] that causes bacterial leaf blight of konjac (*Amorphophallus konjac*). Konjac is grown in East and Southeast of Asia for its large starchy corms, used to create a flour and jelly, and also used as a vegan substitute for gelatin. The major symptoms of the disease are leaf spots and leaf blight, but under severe conditions the petioles become infected, and the plant will wilt and the roots may rot [61]. The bacterium enters the plant through stomatal openings and wounds and spreads by wind, rain and contact with diseased material [62], being able to survive for extended periods of time in plant debris and soil. Recently, it has also been reported that this bacterium causes black rot disease on Korean radish (*Raphanus sativus*) [63], an important vegetable crop in Korea, showing brownish-black symptoms on both exterior and interior of the roots, significantly reducing the value of the agricultural product. It was also reported black spot disease on cucumber (*Cucumis sativus*) with the detection of water-soaked leaves and black spots [64].

A last, more subtle yet emerging member of the *Acidovorax* genus, is *Acidovorax valerianellae*, subject of this Master's dissertation.

2.2.3 *Acidovorax valerianellae*: An emerging pathogen of lamb's lettuce

A. valerianellae causes bacterial black spot of lamb's lettuce (*Valerianella locusta*), also called corn salad [65], a member of the family *Valerianaceae*. In Asia, it was also reported that *A. valerianellae* can infect watermelon [66, 67], tea [68] and hydrangea [69].

Bacterial black spot was observed for the first time in western France fields in 1991 and since then widespread in several other countries in Europe, being responsible for economic losses in corn salad cropping of at least 10% in tonnage every year [70, 71]. Typical disease symptoms appear as black spots on cotyledons, leaves, petioles and stems. Cotyledons and leaves initially develop water-soaked, angular lesions, which later develop into black necrotic spots. In the case of severe infection, foliar lesions may also coalesce into blights [72]. This reduces corn salad quality significantly, making the affected batches unmarketable [71]. The period between infection and symptom expression ranges from 3 to 21 days, and is influenced by temperature, plant age, and soil characteristics, including the nutrient status of the plants. *A. valerianellae* colonizes leaves and roots shortly after seed germination and can be detected even without symptom development [72]. Transmission by contaminated seeds and soil are discussed as major infection sources [73]. Bacteria released from leaf and stem lesions in exudates can also be spread via splashing water and wind driven rain, facilitating short-distance pathogen dispersal, entering the host tissue through natural openings, such as stomata, or wounds possibly caused by agronomic operations [72]. Figure 2.1 shows the dispersal of the disease in the cultivation method of the crop. During the late stages of lamb's lettuce production, when plants are densely planted and overhead watering is used, this poses a major danger for the development of a black spot pandemic. Between

lamb's lettuce plantings, *A. valerianellae* persists in contaminated seeds and infected plant debris in the soil, being able to be recovered from soil for up to 39 days after harvest of a diseased crop [70]. Continuous lamb's lettuce cultivation raises soil inoculum levels, which leads to infection of subsequent crops during germination and other plant growth stages.

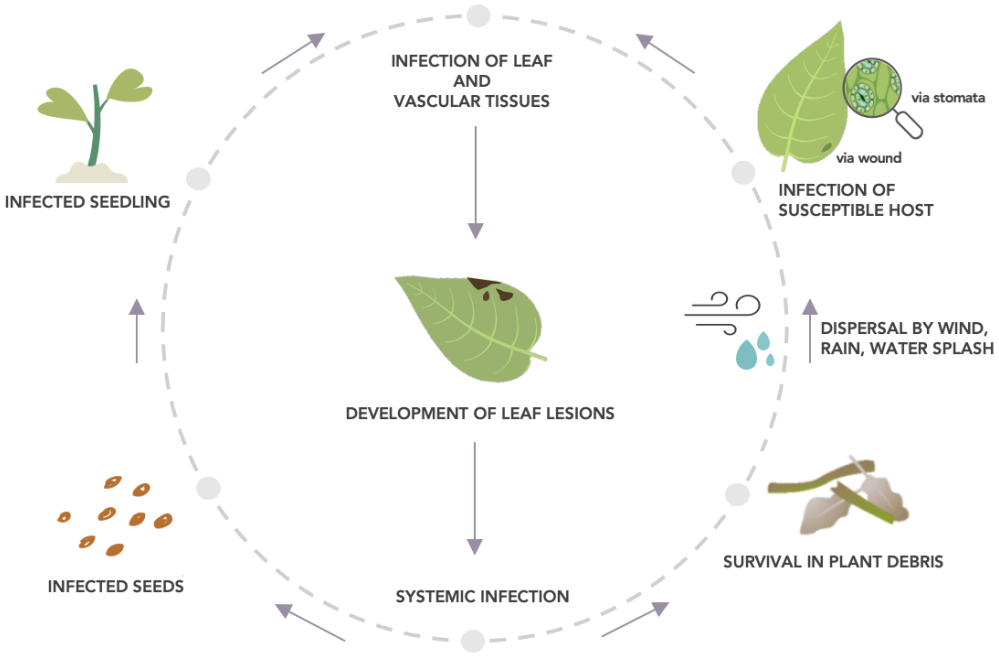

Figure 2.1: Overview of the disease cycle of *Acidovorax valerianellae*. Infection of leaf and vascular tissue in crops can occur through infection of the seed or by external factors. This infection leads to the development of foliar symptoms, which in turn results into the decay of the xylem and ends with a systemic infection of the plant. The pathogen can be further dispersed by contaminated seeds which results into the formation of infected seedlings, or it can survive in a debris-filled soil. Splashing water and wind driven rain facilitates short-distance pathogen dispersal, that can enter other host through natural openings, such as stomata, or wounds possibly caused by agronomic operations. Adapted from [74].

In recent years, fresh-cut vegetables have attracted consumer interest as a practical alternative to traditional vegetable crops presenting a wide number of advantages, such as freshness, safety, convenience, and labeled information [75]. These factors often result in higher prices and so to economic benefits for those involved in the production chain [76]. Therefore, the production of ready-to-eat leafy vegetables have gained increasingly importance in Europe. Lamb's lettuce grows in a low rosette with spatulate dark green leaves up to 15.2 cm long. It is mainly produced for the preparation of ready-to-use salad, but also for traditional sale in trays [20]. The plants grow wild in Europe, northern Africa and western Asia, and has been traditionally cultivated for use in salad during autumn and winter months [72]. In Europe, it is produced mainly in France, Germany and Italy. The Nantes area in western France accounts for 90% of French lamb's lettuce output, which represented 75% of global production in 2000 [20]. Furthermore, lamb's lettuce is among the mostly requested baby-leaves commercialized in the Italian market [77]. For a long time, corn salad was strictly considered a winter salad. Out-of-season (April to September) cultivation is now feasible due to the use of advance cultivation techniques allowing for continuous, year-round production of this leaf vegetable [78]. It is cultivated under plastic tunnels that create high humidity conditions that promote plant growth and, by extension, bacterial disease development [72]. The growing period of corn salad varies according to the weather; harvesting can begin from 40 to 110 days after sowing. Harvesting takes place when 3-4 pairs of leaves for sealed plastic bags, 4-6 pairs of leaves for sales in plastic punnets and 7-8 pairs of leaves for traditional sale in trays [78].

Currently, it is particularly difficult the control of black spot of lamb's lettuce in the field once an

outbreak occurs. To date, no resistant varieties of *V. locusta* or chemicals are available that effectively control the disease under field conditions. Nevertheless, significant efforts to seek for seed treatments have been made [79, 80], and other management strategies have been explored to control *Acidovorax* pathogens.

2.2.4 Integrated pest management of *Acidovorax*

As *Acidovorax* pathogens have shown resistance to antibiotics [13], and because its overuse has been raising public concern, alternative control strategies are eminent.

A wide range of seed treatments, including thermotherapy, chemical and biochemical methods, have been conducted for the control of *Acidovorax* pathogens, such as *A. citrulli* and *A. valerianellae*. However, it remains a big challenge to completely decontaminate the infested seeds, once the pathogen is located in the embryo, where it is protected from external antimicrobial compounds [41, 81]. For instance, fermentation of cucurbitaceous seeds with chitosan, streptomycin sulphate, sodium hypochlorite, peroxyacetic acid, mercury chloride, hydrochloric acid, calcium chloride, have been reported to significantly reduce seed-to-seedling transmission of BFB disease, but unable to fully eradicate *A. citrulli* [41, 81]. In lamb's lettuce, several seed disinfection methods were tested, including aerated steam, hot water, sodium hypochlorite, ethanol and calcium hydroxide [79]. Although sodium hypochlorite revealed to have an effect against *A. valerianellae*, it is not allowed in organic farming since it presents poor degradability and potential toxicity [80].

Steam treatment of the soil has also been used in lamb's lettuce production once a year [72]. Depending on the type of culture, steam is injected into the soil to a specified depth. Pathogens and weeds are killed when the soil temperature reaches 80-85^{o}C, without compromising the physical and chemical soil qualities that are necessary for plant growth [72]. Although superficial soil disinfestation decreases bacterial inoculum it does not totally eradicate pathogen propagules.

Biological control with natural antagonistic microorganisms present in the rhizosphere has been exploited against different plant diseases, among which the *Bacillus* genus is one of the most frequently studied biological control agents. Some attempts have been made to control BFB using *Bacillus*, by developing seed coating formulations and treatments, that significantly reduced BFB seed transmission [82, 83, 84, 85]. For instance, the antagonistic *Bacillus amyloliquefaciens* 54 showed potential in control the BFB by increasing the expression level of defense-related gene PR1 and the accumulation of hydrogen peroxide in the plant [84], and *B. subtilis* 9407 efficiently controlled BFB through the production of surfactin [83]. Also, Masum *et al.* showed the ability of halotolerant bacteria from the group *B. amyloliquefaciens* to inhibit the growth of *A. oryzae*, demonstrating the great potential of biocontrol of the rice brown stripe disease [86].

Fessehaie and Walcott demonstrated that biological seed treatment with a strain of *A. avenae* effectively reduced transmission of infection by *A. citrulli* in growth chamber and green house conditions [87]. Additionally, it was also successful in reducing seed infestation when applied as a protectant to female watermelon blossoms. However, the *A. avenae* strain used in these experiments is pathogenic to maize and therefore could not be commercialized as a biocontrol agent [87].

Another biocontrol approach is based on the generation of nonpathogenic strains of phytopathogens and has been used before to manage plant diseases. These strains can occur naturally or can be generated by mutagenesis. Johnson *et al.* [88] developed a nonpathogenic mutant of *A. citrulli* that was able to reduce BFB seedling transmission.

Some other biocontrol strategies have also been explored to treat BFB, including the use of rhizobacteria [89], yeasts [90] and bacteriophages [42, 91]. The latter will be further described in Section 2.3.4.

2.3 Bacteriophages as a Strategy in Biocontrol

2.3.1 History of Bacteriophages

Bacteriophages are the most abundant biological entity in the biosphere with an estimated number of 10^{31} [92]. Phages are viruses that infect bacteria, subverting the metabolism of their bacterial hosts in order to replicate, and displaying the ability to kill them while not affecting cell lines from other organisms.

Bacteriophages were found out in parallel by Frederick Twort [93] and Félix d'Herelle [94]. With the quick recognition of their potential as antibacterial agents, the application of phages has been proposed since its inception as a therapy to treat acute and chronic infections [95]. Studies were also initiated with the aim of using phages to control plant diseases. Mallmann and Hemstreet (1924) showed the inhibition of *Xanthomonas campestris* pv. *campestris* in cabbage [96], and Kotila and Coons (1925) demonstrated the prevention of soft rot by *Pectobacterium atrosepticum* and *Pectobacterium carotovorum* ssp *carotovorum* on potato and carrot, respectively [97, 98]. The first field trials were also performed by Thomas (1935), who showed the reduction of the incidence of Stewart's wilt disease by treating seeds with phage against the phytopathogen *Pantoea stewartii* [99].

The initial fervor over phage therapy as a treatment for bacterial diseases in the pre-antibiotic era was understandably big, but also highly controversial and not widely accepted by the public or medical community alike. The emergence of age of antibiotic chemotherapy with the introduction of sulfa drugs in the 1930s and later penicillin in the 1940s further dampened enthusiasm on phage research, remaining an active area of research and development only in the former USSR and Poland [95]. However, over the last decade, the west is facing a 'phage therapy revival' brought on by the emergence of multi-drug resistant bacteria that led investigators to re-consider this approach.

2.3.2 The Sequencing Era and Phage Taxonomy

The classification of phages has been a subject of discussion since their discovery with different classification schemes for the taxonomy of phages being proposed over time. Nevertheless, they have been traditionally classified according to their morphology, using electron microscopy images, their genetic content (DNA vs. RNA), their specific host, their habitat and their life cycle [95]. The importance of phage genome sequence comparisons has been recognized more recently. The International Committee on the Taxonomy of Viruses (ICTV) is now in charge of creating an internationally agreed upon viral taxonomy and ratification of newly proposed viral taxa.

The vast majority of the identified phages belong to the order *Caudovirales* [100], proposed in 1998 by Hans-Wolfgang Ackermann, that unifies all tailed phages. For tailed phages, the formal taxonomy adopted in 1971 by the International Committee on Nomenclature of Viruses (ICNV), and later accepted by ICTV in 1981 and 1984, has been classifying them into three morphotypes: *Myoviridae* which have a contractile tail, *Siphoviridae* with a long, non-contractile tail and *Podoviridae* with a short non-contractile tail [101], as illustrated in Figure 2.2. Based on morphology and genetic material, the non-tailed phages have been organized into other 11 distinct phage families [102] (Fig. 2.2), not limited to a single order. For example, filamentous phages have been included in the *Inoviridae* family.

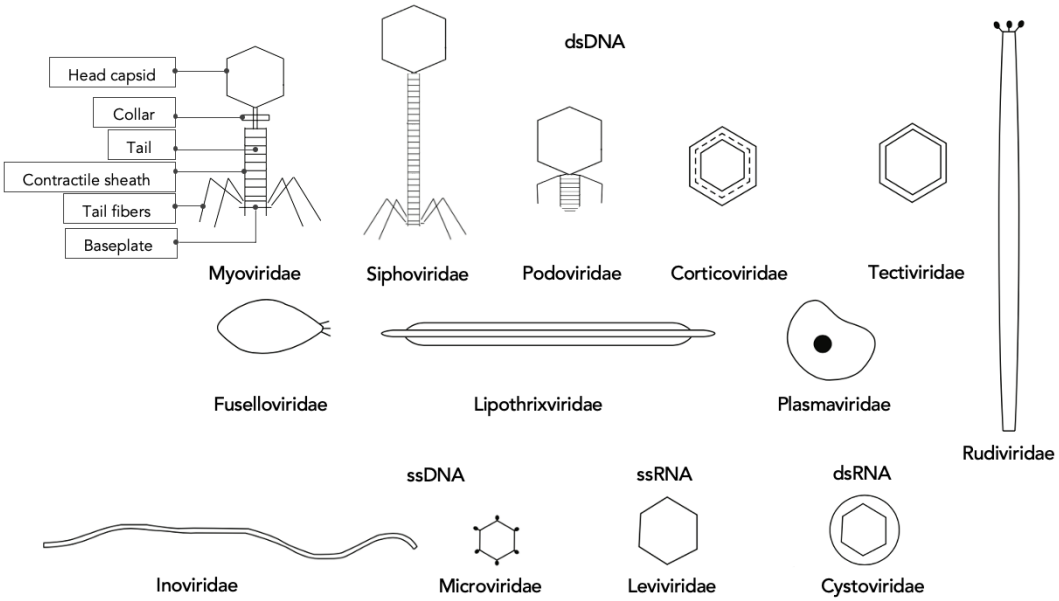

Figure 2.2: Phage families based on morphology and genetic material (ss/ds DNA/RNA) and generalized structure of a tailed phage. Adapted from [102].

With the beginning of the genomic era in the early 2000s, the sequencing of phage genomes showed far more genomic variety than previously thought, particularly in bacteriophages belonging to the order *Caudovirales*. This resulted in a more frequent updating and formation of new families and subfamilies, that today already accounts for 14 different families in the *Caudovirales* [103], and also a higher number of non-tailed phage families, belonging to several different orders.

In order to address the recent increasing genomic diversity, ICTV has been expanding taxonomic ranks and is now proposing some other fundamental changes to classification, including the abolishment of the order *Caudovirales* and of the classical families *Myoviridae*, *Podoviridae*, and *Siphoviridae*, to replace them with monophyletic, genome-based families [101]. Despite this, it is suggested that the terms myovirus, podovirus, and siphovirus continue to be used to describe phage morphology [101]. Specific criteria for ranking tailed phages in the class *Caudoviricetes*, have also been defined. Two phages are assigned to the same species if their genomes are more than 95% identical at the nucleotide level over their full genome length, tested reciprocally. To create distinct genera, 70% nucleotide identity of the full genome length was established as the cut-off. Subfamilies are created when two or more discrete genera are related below the family level. A family is represented by a cohesive and monophyletic group in the main predicted proteome-based clustering tools (ViPTree, GRAViTy dendrogram, vConTACT2 network), and its members share a significant number of orthologous genes. Orders should be proposed when two or more families are related [101].

2.3.3 Life and Infection Cycles

Phages can display different life cycles within the bacterial host: lytic, lysogenic, pseudolysogenic, and chronic infection [95], as illustrated in Figure 2.3.

Independently of the type of cycle of a phage life, the first step in phage infection is the attachment to specific receptors of the bacterial cell wall by specialized adsorption structures, such as fibers or spikes. The type and location of host cell receptors vary considerably, ranging from peptide sequences to polysaccharide moieties, influencing the spectrum of the possible phage-bacteria interactions [95]. More than one receptor may also be involved in the adsorption process, and specific cofactors can be required for binding, such as divalent cations like Ca^{2+} or Mg^{2+} [104]. In Gram-positive bacteria, peptido-

glycan and teichoic acid are often involved in bacteriophage adsorption, while in Gram-negative bacteria cell proteins and constituents of the LPS are usually used [105]. Additionally, other bacterial structures, such as flagella, pili and capsules can also serve as receptors for phages, as described for the *Pseudomonas* phage MP22 that attach to the pili [106] and for *Salmonella* phage iEPS5 that uses a flagellar protein [107]. Bacteria can develop resistance to a specific phage through mutational loss or by altering the receptor [108]. For example, Zaleski *et al*. demonstrated the loss of a lipo-oligosaccharide phage receptor in *Haemophilus influenzae* strains in defense against phage infection [109]. However, losing a specific receptor does not provide protection to other phages binding to other receptors. In addition, phages can acquire compensating adaptations to their receptor binding protein (RBP) which leads to host range mutant phages, mostly a result from alterations in their tail fibers [110]. For instance, *Bordetella* phage BPP-1 can alter its host specificity through a reverse transcriptase-mediated mechanism, introducing a combination of specific mutations in a region upstream of the RBP [111]. Some phages are also able to synthesize specific enzymes (such as hydrolases or polysaccharidases and polysaccharide lyases) that degrade exopolysaccharide structure capsules before the interaction with their specific receptor. This has been described for phage ϕK1-5, that expresses an endosialidase, which allows this phage to attach to and degrade the *E. coli* K1 polysaccharide capsules and a lyase, that specifically cleaves the *E. coli* K5-capsular polymer [112].

Upon irreversible binding, phages induce a pore in the bacterial cell wall and inject its DNA into the cell, while the viral capsid remains outside of the bacteria. Phages protect their DNA from degradation by the host by circularizing the linear DNA or protecting the genome ends. In addition, some phages inhibit the nuclease of the host or protect the genome against nucleases using modified nucleotides or through evolutionary loss of genome sites that would have been recognized [110].

This is followed by the expression of phage early genes, which, in the case of lytic phages, redirects the bacterial synthetic machinery to the reproduction of viral nucleic acids and proteins [95]. The DNA is then packaged into preassembled icosahedral protein shells, procapsids [110]. There are different DNA packaging strategies: single-stranded cohesive ends; circularly permuted direct terminal repeats (“headful packaging”); short direct terminal repeats; long direct terminal repeats; terminal host DNA sequences, and covalently bound terminal proteins [113].

Finally, phages’ late enzymes are employed for cell lysis and virion burst in the extracellular environment. The phage holin creates pores within the cytoplasmic membrane, enabling the phage-encoded endolysin to gain access to the peptidoglycan layer that it hydrolyzes [114]. The number of viral particles released varies depending to several factors including the phage itself, the state of the bacteria host and environmental factors such as nutritive compounds surrounding the host [95].

In the case of lysogenic cycle, temperate phages insert their genetic content into the bacterial chromosome using a phage-encoded integrase enzyme. A specific phage repressor (CI in phage lambda) inhibits the transcription of most of the genes, including those required for the lytic cycle, and the prophage becomes quiescent, remaining silent for extended periods. The presence of several prophages was already identified in many bacterial plant-pathogen genomes, including *Acidovorax citrulli*, *Agrobacterium* and *Burkholderia* [115]. Prophages are replicated as part of the bacterial chromosome during cell division and vertically transmitted to its progeny until the induction of the lytic cycle [114]. Spontaneous prophage induction can be triggered by extrinsic factors, such as DNA damage by reactive oxygen species and UV radiation, and the effects of antibiotics such as mitomycin C and fluoroquinolones, or by intrinsic factors which affect genomic DNA or RecA and induce a SOS response [116].

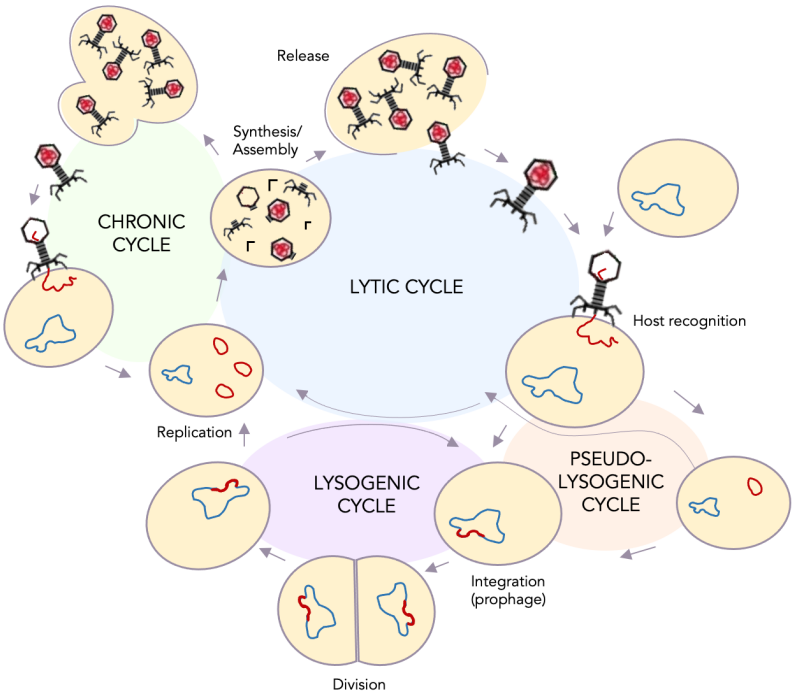

Figure 2.3: Overview of phage life cycles: chronic, lytic, lysogenic and pseudolysogenic cycles. Host-phage interactions range from true non-lethal parasitism (stable coexistence in the chronic cycle) to fatal lytic infection (lytic cycle), with intermediate mutualistic lifestyles (lysogenic and pseudolysogenic cycles). Adapted from [117].

Prophages can impact host fitness in many different ways. In addition to the metabolic cost to replicate extra DNA and the lysis of the host after prophage induction, prophages can also cause host gene disruption upon integration and genome rearrangement by homologous or illegitimate recombination [118]. For example, the integration of the phage PHB09 reduced the virulence of *Bordetella bronchiseptica* through disruption of a gene encoding a pilin protein, a known pathogenicity factor [119]. In another case, the attenuated pathogenicity of *Bacillus anthracis* was found to be associated with a large chromosomal inversion, caused by an internal recombination between homologous regions within two prophages [120]. On other hand, there are also several cases of mutualistic interactions between temperate phages and their hosts. Prophages can provide immunity against lytic infection, which is achieved through different mechanisms, mainly involving alterations to the cell surface or other cell envelope components that inhibit DNA entry [121]. *E. coli* phage HK97 expresses, for instance, a gene that produces a small likely inner membrane protein that inhibits superinfection by HK97 by preventing DNA entry into the cytoplasm [122]. Furthermore, prophages can express a wide variety of adaptive accessory genes that can provide host fitness advantage, augmenting its metabolism, immunity and evolution, or encode toxins or virulence factors that contribute to bacterial colonization and pathogenesis [121]. Varani *et al.* showed that 37 fully sequenced bacterial plant-pathogen genomes carried approximately 5000 genes of phage origin, including virulence factors contributing to pathogenicity and others associated with diversified functions such as cell division, lipid synthesis, transport, conjugation, and plasmid recombination [115]. Moreover, prophages can act as vehicles for horizontal transfer of bacterial genes either by generalized transduction, when the phage packages exclusively host DNA instead of its own, or specialized transduction, in which the packaged DNA is a hybrid piece comprising some phage genome linked to the bacterial DNA [123]. Generalized transduction has been shown to be used as a tool for cooperation between temperate phages with their hosts to survive in rapidly-changing environments [124, 125]. For instance, the ability of lysogens to acquire beneficial genes, such as antibiotic resistance genes, from neighboring cells through autotransduction was demonstrated by Haaber *et al* [125].

Lysogenic phages can also mutate after infecting the bacteria and lose the capacity to induce a lytic replication cycle (chronic infection). The phage DNA becomes a new part of the bacterial chromosome and becomes a long-term prophage sequence. This life cycle is usually associated with the release of phages from the bacteria without causing cell lysis [126].

Another remarkable observation in phage infection, referred to as pseudolysogeny and the phage carrier-state, represents an alternative developmental route, usually triggered by conditions of suboptimal growth or starvation [127]. Here, the viral DNA is carried by the bacteria as a plasmid rather than integrated into the bacterial chromosome, and can be terminated with initiation of either true lysogenization or lytic growth when growth conditions improve [128]. Interestingly, the plasmid is not distributed among all daughter cells during cell division, illustrating that the phage DNA is not copied and passed on to later generations in the bacterial community. This has been shown by Cenens *et al*. with the asymmetrical segregation of an episomal P22 element in *Salmonella typhimurium* [129]. The emergence of pseudolysogeny in response to an infection by a lytic phage in minimally subcultured *P. aeruginosa* was investigated by Latino *et al*., demonstrating its important role in allowing the maintenance of the phage [130, 131]. However, a resulting destabilization of the genome was also observed with a high level of large chromosomal deletions arising during phage-maintenance. No accumulation of phage virions in the pseudolysogens was detected, in contrast to the carrier state established by the levivirus LeviOr01 in *P. aeruginosa*, described in another study, in which large quantities of virions could be observed inside enlarged cells [132].

2.3.4 Application of Bacteriophages in Biocontrol

2.3.4.1 General Considerations

Phage selection

The success of phage control starts with the isolation and characterization of suitable phages. Phage isolation methods from environmental samples are already relatively well established [133]. A representative collection of bacterial strains of the intra pathovar diversity can be used in enrichment techniques to isolate novel phages, which are likely to be present in environmental niches in which the host bacterium is present. Phages in that niche are most likely adapted to the local environmental conditions and bacterial host [134]. Notably, phages were already isolated from highly diverse environments ranging from common sources like soil or sewage to extreme environments such as hot springs [135], glaciers [136], sea ice [137] and saltwater lakes [138]. Despite the large amount and diversity of bacteriophages, finding the one that best suits a particular downstream application often requires many repetitive and time-consuming steps and involves many failed attempts. High-throughput (HTP) approaches have then been arising to carry out phage isolation and analysis in a large-scale and automated manner. Microtiter plates, when combined with robotic liquid handlers and HTP incubators, can continuously process a large number of samples and bacteriophages, performing most of the steps required for phage isolation and characterization such as host range, virulence, growth assays and bacterial resistance development [139, 140]. HTP image analysis can, in turn, be used to increase the rate and accuracy of analysis of phage plaques [141]. For instance, the company Locus Biosciences, Inc. is currently using a high-throughput robotic platform to automate the process of phage discovery [142].

The main deciding factor whether an isolated phage is applicable for biocontrol (and also therapy in humans or animals) is whether the phage is exclusively lytic (virulent) or instead temperate in nature [92]. Since the clarity of a plaque gives an indication of the efficiency at which a phage can lyse its host, phages that produce clear plaques are preferentially selected to reduce the isolation of temperate phages. One of the main concerns with temperate phages is the spread of virulence or antibi-

otic resistance genes by transduction. The mobility and shuffling of phage-borne toxin genes makes them particularly dangerous and facilitates the emergence of novel pathogens by lysogenic conversion, since in some cases the presence of a single toxin gene can make the difference between a harmless and harmful bacteria. For example, upon the integration of the prophage VPIΦ that encodes the toxin co-regulated pilus, *Vibrio cholerae* was converted from a harmless water-dwelling bacteria to a major pathogen [143]. By excluding the use of temperate phages, the possibility of specialized transduction is, in turn, eliminated. In addition, generalized transduction, typically associated with phages that use a headful packaging mechanism [144], can be avoided with the respective phage genomic characterization and identification of the packaging mechanism.

On the other hand, engineering temperate phages to become virulent was demonstrated as a possible alternative strategy for selecting phages for phage therapy. The removal of a repressor gene of a temperate *Mycobacterium smegmatis* phage led to the production of larger and clearer plaques on *Mycobacterium abscessus* and to an increase of the efficiency of plating by 100-fold [145]. This modified phage was then used in the successful treatment of a patient with *M. abscessus* infection.

The suitability of filamentous phages, which chronically infect their host, for biocontrol is also questionable since it has been reported that their infection can have varying effects on host virulence [146]. While some filamentous phages offer an opportunity for environmentally safe biocontrol of pathogens by reducing the pathogenicity of their bacterial host, as shown by Addy *et al.* with ΦRSM in *Ralstonia solanacearum* [147] and by Ahmad *et al.* with XacF1 in *Xanthomonas axonopodis* [148], it was also reported the reverse effect, with the enhancement of the virulence of *R. solanacearum* by the infection of the phage ΦRSS1 [149]. Recently, Akremi *et al.* demonstrated in a bioassay that a cocktail of four filamentous phages can reduce by 40% fire blight symptom development of *E. amylovora* infection in pear [150]. Therefore, besides filamentous phages can be potential biocontrol agents they should be extremely well characterized before use.

Subsequently, to ensure the selected phages are appropriate candidates for biocontrol, *in vitro* characterization of the phages is essential to allow a rational design of a phage cocktail and to enable the tracking of phages during bioassays and field trials. The determination of a broad host range that allows productive infection on all strains of the pathogen species being targeted is desired. Also, it should be able to lyse the host quickly while replicate rapidly producing a high burst size and diffuse easily though the environment [92]. Thereby, basic growth parameters, such as adsorption curves, length of infection and burst size, or stability assays within the conditions likely to be encountered in the field, have been widely performed in the context of phage biocontrol research [151, 152, 153, 154]. Furthermore, identification of the phage receptors can assist in the rational selection of phages that target through different mechanisms to reduce the frequency of resistance [155]. Also, by using mutant bacterial hosts it is possible to enrich for the isolation of phages with alternative receptors [156].

While the traditional characterization by molecular methods and transmission electron microscopy has been essential to the understanding of the phage biology, whole-genome sequencing has arisen as a powerful tool to gain deep knowledge into the phage genome, allowing the identification of genes required for integration and lysogeny or that encode known toxins, antibiotic resistance, or virulence factors [151].

Following phage characterization, bioassays and/or field trials are usually performed to assess the efficacy of the selected phages [151].

Phage persistence in the plant environment

It is crucial to test the persistence of prospective phages in the setting where they will be applied once they have been selected. Although bioassays under laboratory conditions can be very useful

in determining, in a first stage, whether the selected phages are effective for the desired control, the deployment of phages in agricultural systems has shown to be much more challenging given the need to maintain high phage populations on plant surfaces for extended periods of time, as well as the delivery of phages at sufficient quantities to the appropriate sites [157].

The rhizosphere is the area of soil which is in close proximity to the roots of a plant. Several factors of soil structure and chemistry, including pH, moisture levels, presence of organic matter and soil type, have an impact on phage transport and survival of free phages and their hosts [92]. For instance, clay loam soils appear better at maintaining phage at low soil moisture levels and high soil temperatures than that of sandy loam soils [158]. Low soil pH can also negativity affect phage survivability [159]. Usually, soils are only partially hydrated which complicates phage diffusion. Furthermore, phage transport can be limited to biofilms. Phages can also reversible bound to particles such as clays, once these minerals have positively and negatively charged surfaces to which phage can adsorb [160]. Such adsorption can be influenced by pH [161] as well as the presence of organic materials [162]. In general, adsorption limits phage transport, but on other hand can also have a protective effect by keeping them in a hydrated environment [163]. It is reported that if under favorable conditions, phages persist at relatively stable concentrations for several weeks in soil [164].

When bacteria grow, swim or diffuse into phage vicinity or when the soil particle is transferred to the presence of the host bacteria, phage infection can start [165]. A study of the dynamics of the interaction of *Bacillus subtilis* with bacteriophages in soil indicated that an initial phage amplification occurs when bacterial density reaches a critical limit of around 5 x 10^6 CFU/g soil, after which follows a stable equilibrium that can last weeks or months [166]. Disruption of this equilibrium can result in disease.

The phyllosphere, on the other hand, is the portion of the plant which is above the ground and phages can readily be isolated from this location. It can represent, however, a harsh environment for phages to survive, especially during daylight hours. The destructive influence of UV light from the sun has been reported to be a limiting factor for the application of phages for successful biocontrol [167]. Other factors that can cause phage decline on the phyllosphere are desiccation, temperature and pH [168]. Poor persistence on the phyllosphere is then one limitation to effective phage biocontrol on crops. Nevertheless, several methods have been explored to overcome this problem. Avoiding daylight during application might improve phage-based biocontrol [168]. On other side, a range of natural substances that absorb UV and biodegradable polymers were reported to enhance phage performance in the phyllosphere by limiting phage exposure [169, 170]. The application of phages together with UV-protectants was indeed tested in greenhouse trials by Orynbayev *et al.*, in the aim of controlling black rot caused by *Xanthomonas campestris* pv. *campestris* on cabbage. The addition of skimmed milk and riboflavin proved to significantly increase phage survival, rising the phage titer in 6.7 and 5.0 times, respectively, when comparing to control without any UV protector [171]. Additionally, survival of phage in the phyllosphere and rhizosphere can be improved if accompanied by a viable host. This can be an avirulent strain of the pathogen being targeted or indeed another species of bacteria which occurs naturally in that environment. For example, the biological suppression of black rot of broccoli caused by *Xanthomonas campestris* pv. *campestris* was demonstrated in field trials when using a mixture of the bacteriophage XcpSFC211 with a non-pathogenic *Xanthomonas* sp. strain [172]. Given the ability of phages to be systemically translocated inside plants, soil-based phage delivery is suggested as an alternative approach to control foliar plant diseases rather than phage application by foliar spraying. Indeed, Iriarte *et al.* showed that the *Xanthomonas perforans* 97-2 phage mixture reached the upper leaves of a tomato and maintained a leaf tissue concentration of 10^4 PFU/g for seven days, while a typical foliar application would generally drop to undetectable levels within one or two days [157].

Chemical's interference

The combined application of bacteriophages with chemical control has been proposed to establish synergies and decrease the likelihood of resistance evolution [173]. Therefore, the analysis of the possible interactions between bacteriophages and other antimicrobial compounds can be an important part of the development of phage-based products. Despite it has been shown that phages are stable over a range of agrichemicals [174, 175, 176], precautions need to be taken with some combinations of chemicals with phage. Chemical biocides can contain a range of phage inactivating substances such as surfactants and chelators [177, 178]. Also, copper-based bactericides can inactivate some phages, which can be avoided with the delayed application of phage (4–7 days) after initial application of copper-based bactericide [168]. Li *et al.* demonstrated that lipid-containing phages were most susceptible to copper toxicity, whereas most dsDNA phages were unaffected [179]. The combined effects of bacteriophages with systemic acquired resistance inducers have also been studied in plant protection framework. For instance, several combinations of harpin protein and acibenzolar-S-methyl (ASM) with bacteriophages were compared by Obradovic *et al*. in field experiments for the control of tomato bacterial spot caused by the pathogen *Xanthomonas vesicatoria*. In result, reduction in disease pressure and a more efficient foliar disease control were obtained with a combination of ASM and phage [180].

Co-evolution of bacteria and phage

There has always been a constant race between phage and bacteria in nature, and their interaction in soil results in a co-evolutionary process that drives the diversification of both. The role of bacteria-phage coevolution in natural populations has been the focus of a number of studies due to its broad importance in a range of ecological and evolutionary processes, including population dynamics and extinction risk, the evolution of diversity, speciation and mutation rates, and the evolutionary ecology of pathogen virulence. For instance, Gómez *et al.* explored the coevolution dynamics between bacteria and phage in a soil community, which shows differences to coevolution in vitro in infectivity and resistance through time. They also demonstrated that intraspecific competition and parasitism play a more substantial role than interspecific competition in driving evolution within microbial soil communities [181]. In addition, experimental studies by Lopez-Pascua *et al.* [182, 183, 184] have suggested that the level of resources available for hosts shapes the outcome of coevolution, by influencing for instance the cost of mutating receptors.

Like the acquired resistance to chemicals, bacteria can also become resistant to phage infection. However, unlike chemicals, phages are biological entities which can evolve and overcome these biological alterations in their hosts. The proliferation of bacteriophage resistant strains is a natural process associated with spontaneous mutations, often related to modifications in bacteriophage receptors [185]. Bacterial mechanisms to resist phage infection include the prevention of phage adsorption, the blocking of DNA entry, or systems such as Restriction Modification [186], the Altruistic Abortive Infection (Abi) [187] and the CRISPR-Cas [188]. For example, in the phytopathogen *Pectobacterium atrosepticum* different resistance mechanisms were already detected, including mutations in receptors [156, 189] and both Abi [190] and CRISPR-Cas [191] systems.

Even though, the resistance against phage infection is not necessarily a negative development in the context of phage biocontrol, as it frequently compromises bacterial virulence. Phage-resistance mutations are mainly located in the receptors involved in the phage attachment, that in turn are frequently also involved in the bacterial virulence process, such as lipopolysaccharide (LPS) [189], extracellular polymeric substances (EPS) [192], flagella [156] and pili [193]. For example, resistant mutants of *Pectobacterium atrosepticum* containing mutations in LPS [189] and in flagella [156] showed attenuation in virulence. Therefore, the acquirement of resistance is often accompanied by a secondary fitness cost

that can result in reduced disease severity [194]. In addition, phages can also be selected to avoid the other resistance mechanisms, like the Abi system [195] and the CRISPR-Cas [196, 197].

2.3.4.2 Phage Commercialization and Market

Recently, a number of phage biocontrol products have reached the market.

A USA based company Omnilytics, part of Phagelux, was the first to receive registration for their phage-based biopesticide product, Agriphage [198]. This product line contains four commercial products that have been registered as biopesticides by the US Environmental Protection Agency and are commercialized by Certis USA. These bacteriophage cocktails address bacterial speck and spot disease in tomato and pepper, bacterial canker in tomato, fire blight in apple and pear trees and citrus canker in citrus trees [198]. Another phage product approved for use in the USA was developed by the company Otsuka Pharmaceutical, named XylPhi-PD, which contains bacteriophages infecting *Xylella fastidiosa*, the causal agent of Pierce disease of grape [199].

There is a conflict in European legislation regarding the registration of viruses, particularly bacteriophages. Therefore, in Europe, a very limited number of phage-based products is available on the market in the crop production field. A Hungarian company Enviroinvest was authorized to locally sell a phage cocktail, named Erwiphage, for the control of fire blight of apple trees (active against *Erwinia amylovora*) [200] under strict regulations during the spring of 2021. The Scottish company, APS biocontrol, has developed a postharvest bacteriophage-based wash solution (Biolyse) for potatoes tubers, which is to be used for prevention of soft rot disease (specific against *Enterobacteriacea*) during storage [201].

Several pesticide companies are shifting away from investing in conventional pesticides and instead focusing on biopesticides. In 2014, the pesticide market was roughly $56 billion, with biopesticides accounting for just $2-3 billion of that [202]. In 2020, the biopesticides market increased to $5 billion and is projected to reach $11 billion in 2026 [203]. The expansion of the biopesticide business is likely to outstrip that of conventional pesticides in the future. This shift is thought to be the result of increased customer demand for chemical residue-free foods and increased legislation on the use of synthetic pesticides in some parts of the world. Furthermore, many biopesticides are potentially less expensive to develop and bring to market [202]. Nowadays, to develop a new synthetic pesticide, more than $280 million are required, taking nearly 12 years. A biopesticide, on the other hand, costs between $3 and $7 million to develop and takes four years or less to reach the market in the US [204]. Given the rise of biopesticides and their reduced cost and development time, large agrichemical companies are now acquiring biopesticide companies and products to use in integrated programs with their chemicals [204]. With this economic context, one might anticipate increasing engagement in the development of phage biocontrol as a feasible option for crop disease control in the future. Indeed, a review by Holtappels *et al*. showed that there has been a growing number of scientific publications and patenting activity concerning phage biocontrol over the last years. However, non-profit organizations still continue to show a stronger interest when compared to industry [15].

2.3.4.3 Phage Biocontrol of *Acidovorax*

In recent years, a number of major bacterial plant diseases have gained notoriety for phage biocontrol once traditional techniques have proven ineffective or are restricted in particular parts of the world.

Dickeya, Pectobacterium, Xanthomonas, E. amylovora, R. solanacearum, P. syringae and *Xylella fastidiosa* are the most common crop pathogens where phage biocontrol has been studied and has shown promising outcomes [92]. Despite the growing evidence of the benefits of phage application in several plant diseases in different crops, phage biocontrol studies in *Acidovorax* pathogens have hardly been reported.

Rahimi-Midani and Choi demonstrated the potential of phage biocontrol for controlling Bacterial Fruit Blotch (BFB) of cucurbit crops. Novel bacteriophages for *A. citrulli*, named as ACP17 [91] and ACPWH [205], were fully characterized and tested by both seed coating [205] and soil-based [206] plant assays. Coating of watermelon seeds with the *Myoviridae*- and *Siphoviridae*- family bacteriophages ACP17 and ACPWH enhanced plant germination and survival, modulating the progression of BFB [206]. In particular, the phage ACPWH, that has a wider host range than ACP17, showed a germination rate of up to 90%, in the presence of *A. citrulli*, in contrast to untreated seeds, where no germination or germinated juveniles with BFB symptoms were observed [42]. Although these phages proved to be effective for seed coating and, thereby, prevention of BFB at an early stage, BFB can develop at a later stage due to contamination from soil, workers, and tools and can infect the foliar part. Therefore, ACPWH ability to control BFB by soil application was tested, including the evaluation of the absorption and translocation from soil to the top part of plants [206]. The phage was detected in various parts of plants 8 h following addition to soil, and its abundance increased thereafter. As a result, the melon plants treated with phage ACPWH showed only 20% disease severity, compared to 80% in the control, and the symptoms did not progress [206]. Therefore, this study demonstrated that the application of phage in the soil can effectively treat the infection at a later stage of plant growth.

As seen in the Section 2.2, the current techniques for controlling *Acidovorax* diseases are insufficient, and the abuse in use of pesticides has raised public concern, making biological control measures even more important. The success shown by Rahimi-Midani and Choi in controlling *A. citrulli* with phages indicates that they might be a useful and low-cost biocontrol tool also for other *Acidovorax* infections.

Chapter 3

Materials and Methods

3.1 Bacterial Strains and Growth Conditions

The bacterial strains used in this study, supplied by the Institute for Agricultural and Fisheries Research (ILVO, Belgium), are listed in Table 3.1. The bacteria were grown at 25°C in Lysogeny Broth with medium salt concentrations (LB_{ms}) (10 g/L Trypton (Neogen), 5 g/L yeast extract (Neogen) and 1.5 g/L NaCl (Acros Organics)), while shaking at 200 rpm. LB_{ms} was supplemented with 1.5% agar (bacteriological agar (Neogen)) for plating and with 0.5% agar for agar overlays. For long term storage at -80°C, cell stocks were prepared by adding glycerol (Acros Organics) to an overnight culture to a final concentration of 20%.

3.2 Phage Manipulations

3.2.1 Bacteriophage Isolation, Amplification, and Purification

Phages were isolated from soil samples received from Proefcentrum voor de Groenteteelt (PCG), Proeftuin Sint-Katelijne-Waver (PSKW) and Inagro, from Flanders, Belgium. To enrich for phages, overnight cultures of all bacterial strains (Table 3.1) were grown in 1 mL LB_{ms} at 25°C in 96-deep-well plates and around 1 g of each soil sample was added in each well, following the layout described in Figure 3.1. After overnight incubation, 1 drop of chloroform was added in each well and incubated for 1h. The mixture was then centrifuged (30min, 3000rpm, 4°C), using a Sorvall Legend RT+ centrifuge (Thermo Scientific), and 3 µL of the supernatant was spotted on a soft agar layer that contained the bacterial host. Lysis zones were picked up with sterile toothpicks and suspended in 100 µL phage buffer (10 mM Trizma base (Sigma Aldrich); 10 mM $MgSO_4$ (Sigma Aldrich); 150 mM NaCl (Acros Organics); pH 7.5). These suspensions were plated by pooling 250 µL overnight bacterial host culture, 100 µL phage suspension and 4 mL LB overlay agar. After overnight incubation at 25°C, single plaques were picked up again. Three successive single plaque isolations were performed to achieve pure phage isolates.

Table 3.1: Bacterial strains used in this study, their year of isolation and geographical and biological origin. [a] GBBC: culture collection of plant pathogenic bacteria at ILVO; CFBP, Collection Française de Bactéries Phytopathogènes; LMG, Belgian Coordinated Collections of Microorganisms at the Laboratory of Microbiology of Ghent University with [T] as type strains.

Species and Strain [a]	Year of isolation	Geographical origin	Biological origin
Acidovorax anthuri			
CFBP 3232[T]	1991	Martinique	*Anthurium*
Acidovorax oryzae			
CFBP 2426[T]	1963	Japan	*Oryza sativa*
Acidovorax citrulli			
LMG 5376[T]	1977	USA	*Citrullus lanatus*
Acidovorax cattleyae			
LMG 5286[T]	1961	USA	
GBBC 705	2000	Belgium	*Phalaenopsis*
GBBC 1100	2011	Belgium	*Phalaenopsis*
GBBC 1148		Belgium	*Phalaenopsis*
GBBC 1149		Belgium	*Phalaenopsis*
GBBC 1303		Belgium	*Phalaenopsis*
Acidovorax valerianellae			
CFBP 6945	2006	France	*Valerianella locusta* seeds
GBBC 3037	2015	Belgium	*Valerianella locusta*
GBBC 3038	2015	Belgium	*Valerianella locusta*
GBBC 3039	2015	Belgium	*Valerianella locusta*
GBBC 3042	2015	Belgium	*Valerianella locusta*
GBBC 3043	2015	Belgium	*Valerianella locusta*
GBBC 3129	2016	Belgium	*Valerianella locusta*
GBBC 3161	2016	Belgium	*Valerianella locusta*
GBBC 3208	2017	Belgium	*Valerianella locusta* cv. Calarasi
GBBC 3209	2017	Belgium	*Valerianella locusta*
GBBC 3340	2019	Belgium	*Valerianella locusta*
GBBC 3341	2019	Belgium	*Valerianella locusta*
GBBC 3342	2019	Belgium	*Valerianella locusta*
GBBC 3353	2019	Belgium	*Valerianella locusta* cv. Trophy
GBBC 3354	2019	Belgium	*Valerianella locusta* cv. Trophy
GBBC 3355	2019	Belgium	*Valerianella locusta*
GBBC 3356	2019	Belgium	*Valerianella locusta*
GBBC 3357	2019	Belgium	*Valerianella locusta*
GBBC 3358	2019	Belgium	*Valerianella locusta*

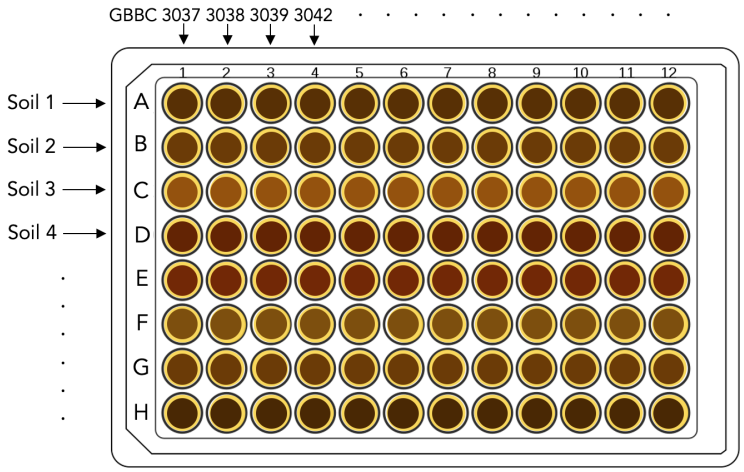

Figure 3.1: Layout of the enrichment with soil samples in 96-deep-well blocks. In each row a different soil sample was added. In each column, a different strain of *Acidovorax* from Table 3.1 was grown in LB and incubated overnight.

Phages were amplified by infecting a liquid culture (in LB supplemented with 10 mM $CaCl_2$ and 5 mM $MgSO_4$) of the respective bacterial host at OD_{600} of 0.3 with a multiplicity of infection (MOI) of 0.01. For Alfacinha1 and Aval the bacterial host used for amplification was GBBC 3357, while for Alfacinha3 this was GBBC 3161 and for Acica was GBBC 1148. After overnight incubation, the supernatant was filtered over a 0.45 µm pore size filter (Millex-HV; Merck Millipore Ltd.). To obtain a phage stock for downstream experiments, polyethylene glycol (PEG_{8000}) (Acros Organics) was added to the filtered phage lysate to a final concentration of 30% v/v. After overnight incubation at 4°C, phages were precipitated by centrifugation (30 min, 4000 rpm, 4°C, Sorvall Legend RT+ centrifuge, Thermo Scientific) and the pellet was dissolved in 2 mL phage buffer.

3.2.2 Electron Microscopy

Transmission electron microscopy (TEM) pictures were made by Dr. Marta Vallino (IPSP-CNR, Turin). In short, the phage suspensions adsorbed for 3 min on carbon and formvar-coated copper-palladium grids, which were then rinsed several times with water. The grids were negatively stained with aqueous 0.5% uranyl acetate and the excess fluid was removed with filter paper. Observations and photographs were made with a Philips CM10 transmission electron microscope (TEM) (Eindhoven, The Netherlands) at 80 kV. Micrograph films were developed and digitally acquired at high resolution with a D800 Nikon camera. Finally, the images were trimmed and adjusted for brightness and contrast using the Fiji software [207].

3.2.3 Host Range Analysis

To test the susceptibility of all the *Acidovorax* strains (Table 3.1) to each phage the double agar overlay method was used, by adding 250 µL of an overnight culture to 4 mL of LB soft agar and poured on top of an LB agar plate. After that, 3 µL of each phage (at least 10^6 PFU/mL) were spotted onto the solidified overlays. After overnight incubation at 25°C, the plates were examined for plaques.

3.2.4 Adsorption and Infection Curves

In adsorption assays, the host strain GBBC 3161 was grown to an OD_{600} of 0.3 and infected with Alfacinha3 at MOI of 0.01. Immediately after infection, a 200 µL sample was taken and transferred into a Zymo-Spin IC column (Zymo Research) in a pre-cooled eppendorf tube and centrifuged for a few seconds. The filtered suspension, kept on ice, was titrated to determine the amount of non-adsorbed or reversibly adsorbed phages. This was repeated after 1, 5 and 10 minutes.

Killing curves were established for the strain GBBC 3161 infected with Alfacinha3 at MOIs of 0.1, 1 and 10, and for the strain GBBC 3357 infected with Alfacinha1 at MOIs of 0.1 and 1, and Aval at MOI of 0.1. The bacterial culture was initially infected at OD_{600} of 0.3, and monitored every 10 min for 2h (GBBC 3161) and 3h (GBBC 3357) and compared with that of an uninfected culture. OD results are the average of three independent biological repeats.

3.3 Lysogeny Assessment

To screen for lysogenic behavior in Aval a phenotypic test was performed following the protocol described in [208], with small differences, as shown in Figure 3.2. First, the phages were spotted (10 µL) in a bacterial lawn and incubated for 3 days. Aval was spotted in 2 different host strains, GBBC 3357 and GBBC 3043. Three turbid plaques were scraped and streaked in new LB_{ms} agar plates, and incubated until the growth of clearly isolated colonies. Streaking of new colonies in fresh LB_{ms} agar plates was repeated three successive times. Nine different colonies from GBBC 3043 and six from GBBC 3357, each three from a different original turbid plaque, were inoculated in 4 mL LB_{ms}. The patch plate assay described in [208] was performed by spotting the liquid cultures of the possible lysogens on the wild-type host lawn (5 µL), instead of streaking the colony. Spontaneous release of phage was assessed by the presence of a halo around the spot after overnight incubation. For the supernatant assay, the cultures were centrifuged (10 min, 4000 rpm) and the supernatant was spotted in the wild-type host lawn (5 µL). After overnight incubation, it was checked for lysis in the spot zones. Finally, in the immunity assay, phages were spotted in the bacterial lawns of the possible lysogens (5 µL) to check for resistance. In the case of the lysogens of the strain GBBC 3357, all phages Alfacinha1, Alfacinha3 and Aval were used to screen for superinfection exclusion, while the lysogens of the strain GBBC 3043 were only spotted with Aval.

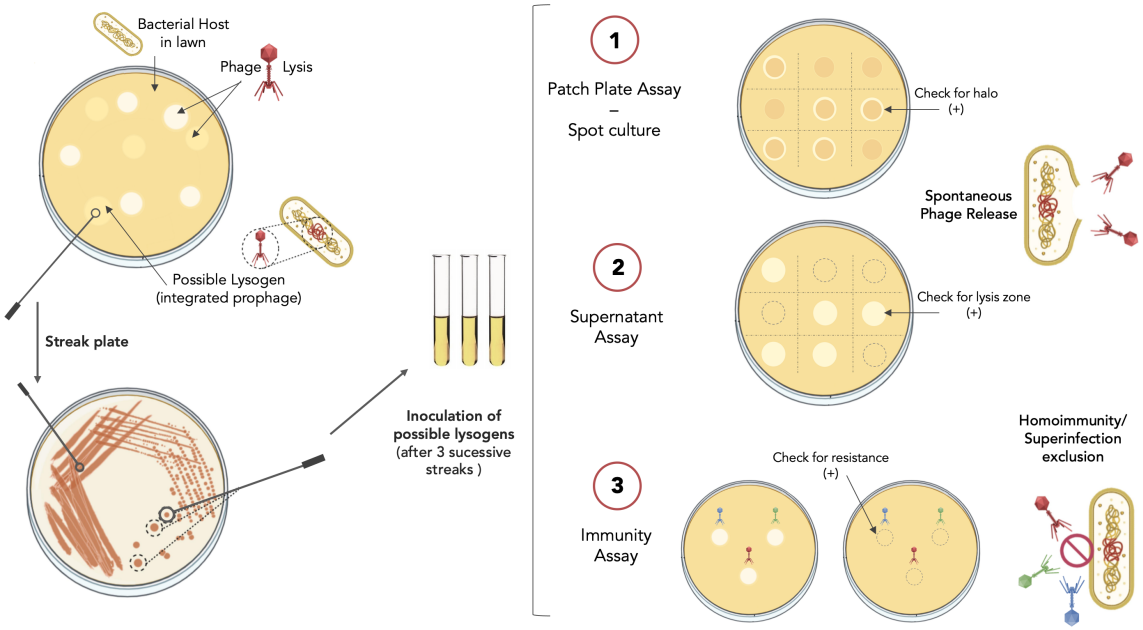

Figure 3.2: Screening pipeline for lysogen activity of a given phage. First, the phage for which the lysogenic behavior is being tested is spotted on a bacterial host lawn and incubated until the formation of turbid plaques or mesas (zones of confluent bacterial growth in the center of the lysis spots). A turbid plaque (or a mesa) is scraped with a sterile wire loop and used as the primary inoculum for streaking a fresh LB agar plate, repeating for each turbid plaque. The plates are incubated until the growth of clearly isolated colonies. The new colonies are picked up and streaked in new LB agar plates for 3 successive times, to remove any phages present. Each different colony, that possibly contain the integrated prophage, is inoculated in a glass tube with 4 mL of LB media, and incubated overnight. The cultures are used in three different assays. In 1 - Patch Plate Assay and 2 - Supernatant Assay, the spontaneous release of phages is tested, by spotting in the bacterial host lawn either the inoculated cultures (1) or the supernatant after centrifuging the cultures (10 min, 4000 rpm, 4°) (2). The plate can be divided into a grid with as many sections as colonies to be screened. The presence of a halo (1) or a lysis zone (2) are assessed. In 3 - Immunity Assay, the inoculated cultures are used as bacterial lawn and homoimmunity or superinfection exclusion are tested by spotting different phages of interest and checking for resistance. Adapted from [208].

3.4 Proteome Analysis

3.4.1 Protein Isolation

The phage proteins were isolated through methanol-chloroform extraction. 100 µL of phage PEG stock (at least 10^7 PFU/mL) were mixed with 450 µL of phage buffer, 500 µL of ice-cooled methanol (Acros Organics) and 375 µL of chloroform (AnalaR), and then centrifuged (5 min, 13000 rpm, 4°C) in a Microfuge 22R (Beckman Coulter). The top phase was discarded without removing the white interface. Following new addition of 500 µL of methanol, it was again centrifuged (5 min, 13000 rpm, 4°C), the supernatant was discarded and the pellet was dried.

3.4.2 SDS-PAGE

The isolated proteins were suspended in 50 µL SDS-PAGE loading buffer (1% SDS, 6% sucrose (Acros Organics), 100 mM 1,4-dithiothreitol (DTT, Roche Applied Science), 10 mM Tris (Acros Organics), pH 6.8, 0.0625% bromophenol blue). After boiling for 5 min at 95°C, samples were loaded on a 12% polyacrylamide gel for electrophoresis. Gels were stained with SimplyBlue™ Safestain (Invitrogen™, Life Technologies).

3.5 Genome Analysis

3.5.1 DNA Extraction and Sequencing

Phage DNA was extracted from a high-titer lysate (minimum of 10^8 PFU/mL). 1 µL of DNaseI (ThermoFisher Scientific) and 1 µL of RNaseA (ThermoFisher Scientific) were added to 10 µL of the phage stock. After incubation at 37°C for 30 minutes, 4 µL of EDTA (Acros Organics), 5 µL of SDS 10% (Acros Organics) and 1 µL of ProteinaseK (ThermoFisher Scientific) were added, and then incubated at 56°C in a thermal bath for 45 minutes. The Kit DNA Clean & Concentrator™-5 (Zymo Research) was used to purify the phage DNA following the manufacturer's instructions.

The genomic DNA was sequenced using Illumina MiniSeq platform at the Laboratory of Gene Technology, KU Leuven. A library was prepared using the Nextera™ Flex DNA Library Kit for each sample, according to the manufacturer's guidelines. The quality of each library preparation was controlled using an Agilent Bioanalyzer 2100. All library preps were equally pooled and sequenced using a MiniSeq Mid Output flowcell (300 cycles; 2*150 bp reads). The reads were trimmed with the Trimmomatic tool (v0.36.5), using standard settings with the addition of an initial ILLUMINA CLIP step to remove the Nextera adapters [209]. Next, the quality of the reads was assessed using the FastQC tool (v0.11.8) [210].

3.5.2 Data Processing and Analysis

The raw read data was processed (assembly and annotation) using online tools on the public servers of Galaxy (v21.05) [211] and PATRIC (v3.6.9) [212]. The reads were assembled using Unicycler (v0.4.8) [213] or SPAdes [214]. The assembled contigs were visualized and their quality was assessed using Bandage (v0.8.1) [215]. To access the variation of coverage between the sequenced reads and assembled sequence, Bowtie2 (v2.4.2) was used for the alignment [216], using Unipro UGENE (v38.1) for visualization [217]. The automated annotation was manually curated by verifying the translated ORFs in a BLASTp analysis ((National Centre for Biotechnology Information (NCBI)) [218] against the non-redundant GenBank protein database [219], and Artemis (v18.1.0) [220] was used to polish the genbank files. To classify the phages based on genome-wide similarities, a viral proteomic tree was generated with the online ViPTree server (v1.9) [221] (accessed in April 2021), and the intergenomic distances/similarities amongst the related viral genomes were computed using the VIRIDIC web tool [222]. Seaborn Python library (v0.11.1) [223] was used for the construction of the heatmaps. Easyfig (v2.2.2) [224] was used to create linear comparison figures of multiple genomes and BLAST comparisons between multiple genomic regions. MegaX (v10.2.4) [225] was run to conduct automatic sequence alignments, using the MUSCLE algorithm [226], and to construct phylogenetic trees according to the neighbor-joining method with 1000 bootstraps.

3.6 Bioassays

3.6.1 Seed bioassay *in vitro*

Valerianella locusta seeds (Groene van Cambrai (Aveve)) were first sterilized by suspending and shaking them for 7 minutes in a 1% NaClO solution. Next, the seeds were rinsed three times with sterile mQ water and left to dry under a laminar flow. The surface sterility of the seeds was tested on LB_{ms} agar plates. The seeds were infected with GBBC 3161 with OD_{600} = 0.15 (around 10^8 CFU/mL) and incubated for 1h30, while shaking using the HulaMixer™ Sample Mixer (Thermo Scientific) at 25 rpm

and room temperature. Following seed drying, Alfacinha3 phage solution was added to the seeds with a concentration of 10^9 PFU/mL and shaked overnight using the HulaMixer^{TM} Sample Mixer at 25 rpm at 16ºC. The seeds were crushed and suspended in phage buffer. The bacterial concentrations were quantified by plating them on LB_{ms} agar plates. The quantification of phages was done by using soft agar overlay method. Overall, three independent repeats were performed for each one of the 4 different conditions (negative control, phage only, bacteria only and bacteria plus phage).

3.6.2 Seedlings bioassay *in vitro*

Similarly as previously described, lamb's lettuce seeds (Aveve) were surface sterilized, infected (OD_{600} = 0.15) and phage primed. After priming, the seeds were dried under a laminar flow. Using sterilized tweezers, 30 seeds per condition were then sown onto plant growth medium, 1/4 MS Agar (1.1 g/L Murashige and Skoog basal medium (Sigma Aldrich); 15 g/L bacteriological agar (Neogen)), in each plate. The plant growth medium enclosed half of the plates so that the seedling started growing parallel to the plate bottom. Furthermore, the plates were sealed with Parafilm (Sigma-Aldrich) to keep the moisture in, and set vertically under a lamp providing light for 16 hours and 8 hours of darkness, in a room maintained at 16 ºC. For each condition, three independent repeats were performed, and so, 90 seeds of each condition were sown in total. After 22 days, the shoot and root length of each seedling was measured using a caliper.

Statistical analyses were performed with JMP Pro 15. Multiple non-parametric Wilcoxon comparison tests were performed at a significance level of 0.05. For statistical data visualization, such as the design of the boxplots, Seaborn (v0.11.1) was used [223].

Chapter 4

Results and Discussion

4.1 Isolation of Novel Bacteriophages

Phages were first isolated from soil samples taken in Flanders (Belgium) from infected lamb's lettuce beds. Using an enrichment, three novel phages were discovered infecting *A. valerianellae*. The first isolate showed small plaques (2-3 mm) with a bull's eyes morphology and was called Alfacinha1 (*Alfacinha* is the Portuguese word for "little lettuce", with lettuce being the main target of *A. valerianellae* and the focus of this study; *Alfacinha* is also popularly used to designate the natives of Lisbon, including the author of this thesis). The other isolates found were Aval (referring to *Acidovorax valerianellae*) and Alfacinha3, showing very small ($<$2 mm) and small (2-3 mm) plaques, respectively. One additional phage, also presenting very small plaques ($<$2 mm), was discovered later for *A. cattleyae*, from samples collected from soil surrounding orchids, and was named Acica (referring to *Acidovorax cattleyae*). Figure A.1 in Appendix A shows the plaque morphologies of all four phages.

The isolate of Alfacinha1 was found by enriching soil samples with *A. valerianellae* strain GBBC 3357, Aval with GBBC 3043, Alfacinha3 with GBBC 3161 and Acica with GBBC 1148. These strains were used to optimize phage amplifications, with the exception of Aval, that was amplified with GBBC 3357 instead of GBBC 3043, since the latter did not allow to obtain concentrations higher than 10^5 PFU/mL. Alfacinha3 was the only phage that could be easily amplified at high concentrations reaching 10^{11} PFU/mL, while for Alfacinha1, Aval and Acica, titers of 10^9 PFU/mL, 10^8 and 10^7 PFU/mL PFU/mL were obtained, respectively.

Transmission Electron Microscopy (TEM) was employed to image the morphology of the phages and estimate their sizes. As shown in Figure 4.1, the four phages exhibited typical head and tail morphologies associated with the *Caudovirales* order. The micrographs showed particles with icosahedral heads and long, contractile tails, typical of a myovirus morphology. The head diameters ranged from 60 nm (Aval and Acica) to 110 nm (Alfacinha1), with Alfacinha3 having a head diameter of 75 nm. The tail length varied from 140nm in Acica and Aval to 135 nm in Alfacinha1 and 125 nm in Alfacinha3. In all cases the tail width was around 23 nm, except in Alfacinha1 where it was considerably narrower (15 nm). Interestingly, the tail of Alfacinha1 exhibited convoluted fibers, being therefore clearly distinguishable from the other phages. Also, both Aval and Acica showed a large amount of empty capsids, revealing the presence of several inactive phages in the stock.

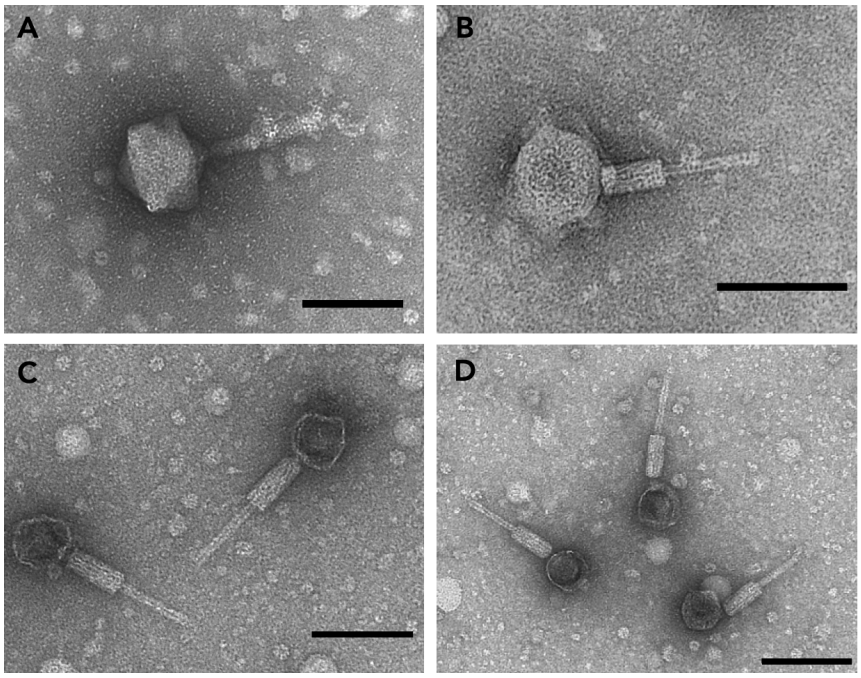

Figure 4.1: Transmission Electron Microscopy images of the phages Alfacinha1 (A), Alfacinha3 (B), Aval(C) and Acica (D). The scale bar represents 100 nm. Phages negatively stained with 0.5% uranyl acetate.

4.1.1 Host Range

The host specificity of a phage is an important factor to consider for its use in biocontrol. To test the host range of the four phages, all the *Acidovorax* strains in collection (Table 3.1) were screened for susceptibility, and the results are presented in Table 4.1.

Aval has the narrowest host range, limited to three *A. valerianellae* strains, while Alfacinha1 has the broadest one, being able to infect not only strains of the *A. valerianellae* species but also strains from the *A. cattleyae* species. Alfacinha3 infects seven different strains of *A. valerianellae* and Acica infects solely *A. cattleyae*. The strain GBBC 3357 could be infected by all three *A. valerianellae* phages, Alfacinha1 and 3 and Aval. In total, 57% of the collection of *Acidovorax* strains could be infected by at least one of the phages, corresponding to 63% of the *A. valerianellae* strains and 67% of the *A. cattleyae* strains. *Acidovorax anthuri, oryzae* and *citrulli* strains were not infected by any of the isolated phages.

Table 4.1: Host range analysis of the phages Alfacinha1, Alfacinha3, Aval and Acica.

Species	Strain	Alfacinha1	Alfacinha3	Aval	Acica
Acidovorax anthuri	CFBP 3232	-	-	-	-
Acidovorax oryzae	CFBP 2426	-	-	-	-
Acidovorax citrulli	LMG 5376	-	-	-	-
Acidovorax cattleyae	LMG 5286	-	-	-	-
	GBBC 705	+	-	-	+
	GBBC 1100	-	-	-	-
	GBBC 1148	+	-	-	+
	GBBC 1149	+	-	-	+
	GBBC 1303	+	-	-	+
Acidovorax valerianellae	CFBP 6945	-	+	-	-
	GBBC 3037	+	-	-	-
	GBBC 3038	-	-	-	-
	GBBC 3039	+	-	-	-
	GBBC 3042	-	-	-	-
	GBBC 3043	-	-	+	-
	GBBC 3129	+	+	-	-
	GBBC 3161	-	+	-	-
	GBBC 3208	+	-	-	-
	GBBC 3209	-	+	-	-
	GBBC 3340	-	-	-	-
	GBBC 3341	-	-	+	-
	GBBC 3342	-	+	-	-
	GBBC 3353	+	+	-	-
	GBBC 3354	-	-	-	-
	GBBC 3355	-	-	-	-
	GBBC 3356	-	-	-	-
	GBBC 3357	+	+	+	-
	GBBC 3358	-	-	-	-

4.2 Aval - an Orphan Temperate *A.valerianellae* Phage

4.2.1 Genomic Analysis

Whole genome sequencing of bacteriophage Aval revealed a genome of 39,584 bp and a GC content of 66.1%. After assembling, the location of the physical start of the phage genome was determined by identifying the packaging strategy. By aligning the sequencing reads against the assembled sequence, it was possible to compare the differences in coverage over the genome. A sudden drop in fold coverage in a unique precise location (*cos* site) revealed the presence of *cos* ends (Figure 4.2). Thus, before genome annotation, the start of the phage genome sequence was first adjusted to begin right after the *cos* site.

Figure 4.2: Variation in coverage of the sequencing reads over the genome of Aval. The square identifies the drop in coverage, indicating the location of the *cos* site.

This packaging strategy was also confirmed by comparing the large terminase of Aval with the sequences from several other phages, whose packaging mechanisms and physical ends were experimentally determined. Figure 4.3 shows the neighbor-joining tree generated from the alignment of the protein sequences of the large terminase from different phages, including Aval, as described by Merrill and colleagues [227].

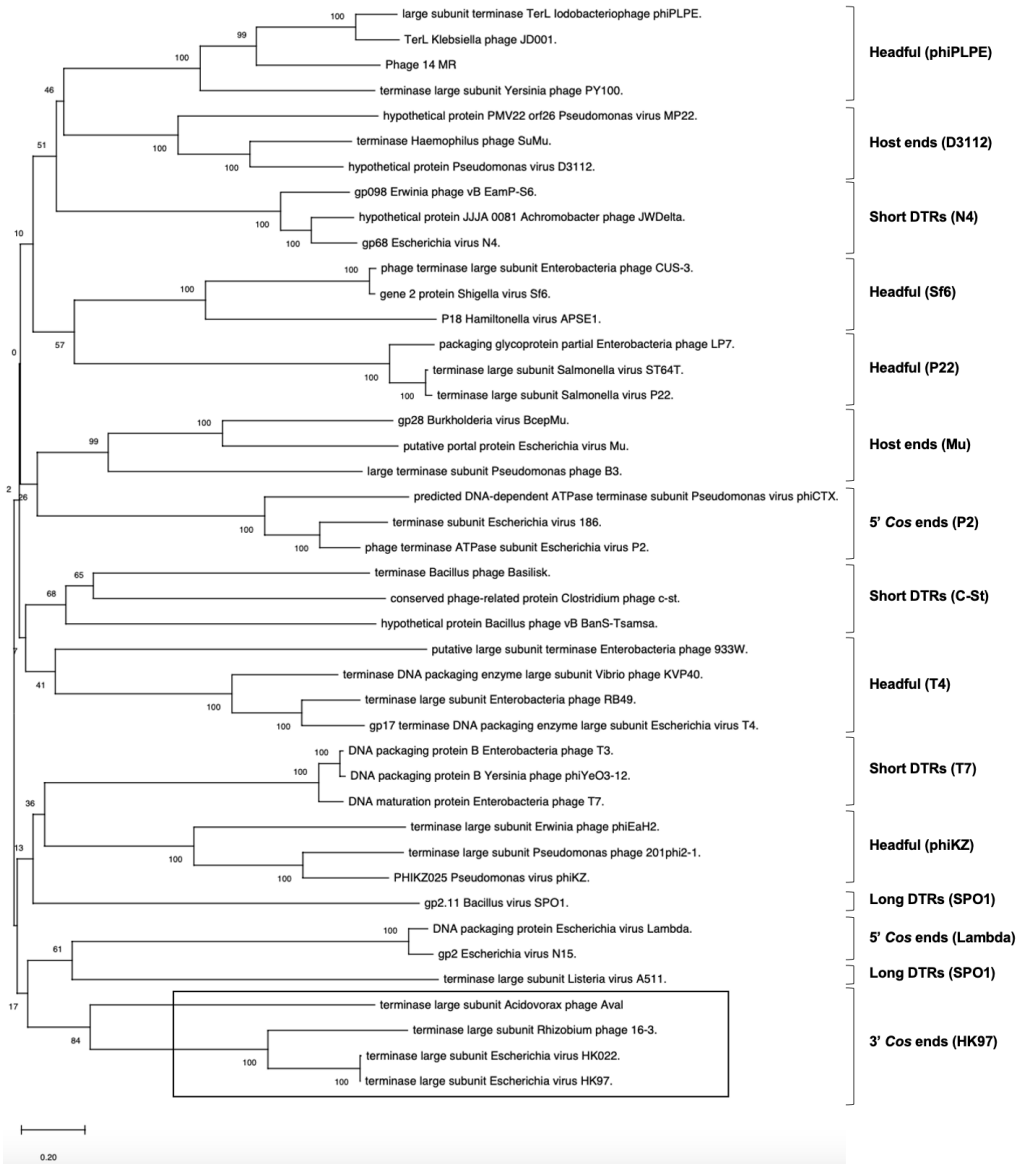

Figure 4.3: Neighbor-joining tree of large terminase protein sequences of Aval and phages whose packaging strategy is known. The black rectangle indicates Aval's cluster. The brackets indicate the packaging strategies experimentally determined for each cluster, according to Merrill *et al.* [227]. Bootstrap values are for 1000 trials. The scale bar shows 0.2 amino acid substitutions per site. Generated in MegaX.

As indicated in Figure 4.3, Aval's large terminase clustered together with the ones from the *Enterobacteria* phages HK97 and HK022 and with the *Rhizobium* phage 16-3, that present 3' *cos* ends [227].

In order to investigate the viral taxonomy of Aval and its similarity with other known phages, a pro-

teomic tree based on genome-wide sequence similarities was generated with the ViPTree server [221] (Figure 4.4), and the pairwise intergenomic distances/similarities amongst the related viral genomes were computed using VIRIDIC [222]. A heatmap based on the calculated intergenomic similarities was constructed and is shown in Figure 4.5.

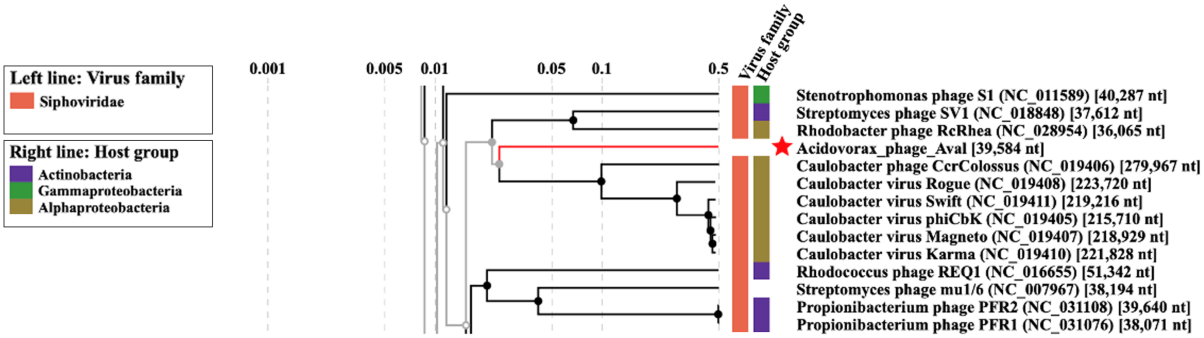

Figure 4.4: Proteomic tree based on global genomic similarity relationships between Aval and other known phages, predicting the virus family and host group. Only the section with the most related phages of the tree is presented in this figure. Aval is indicated with a red star. Generated in ViPTree server.

In the presented section of the proteomic tree (Figure 4.4), which includes only the phages most closely related to Aval, Aval clustered together with phages of the *Siphoviridae* family, mainly with *Caulobacter* phages. However, it has less than 5% of similarity with the phages of its cluster, thus representing a novel species and genus based on the current ICTV guidelines [228].

In the heatmap presented in Figure 4.5 it is possible to distinguish three main clusters, one composed by the *Streptomyces* phages SF1, SF3 and VWB, other with the *Propionibacterium* phages PFR1 and PFR2, and the third one with the *Caulobacter* phages CCrColossus, CCrRogue, CCrSwift, phiCbK, CCrKarma and CCrMagneto. Aval does not belong to any cluster and does not show any homology with any known phage, thus representing an orphan phage genus.

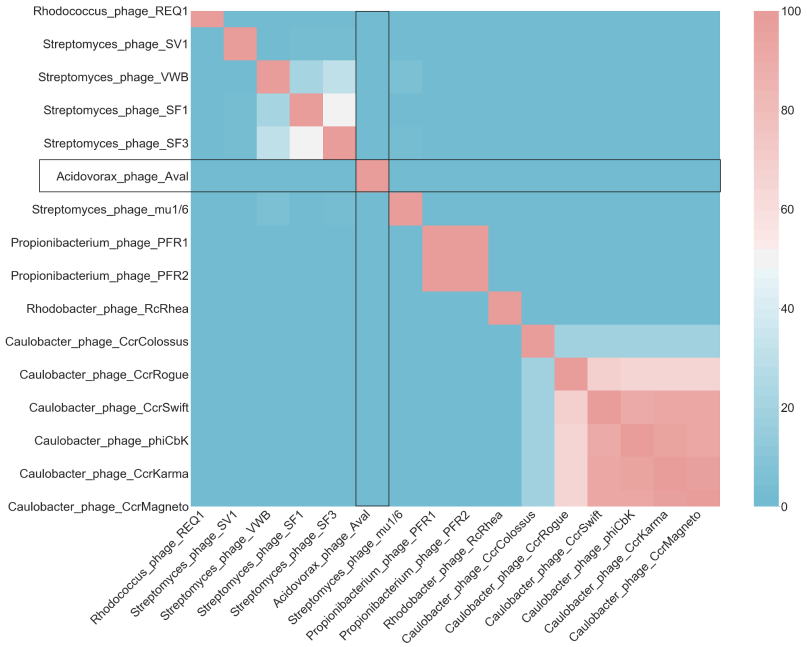

Figure 4.5: Heatmap based on the calculated sequence similarities (using VIRIDIC) between Aval and their most related phages.

Phylogenetic analyses were performed to further explore and characterize Aval. A neighbor-joining

tree of the major capsid protein, a highly conserved protein, is presented in Figure 4.6.

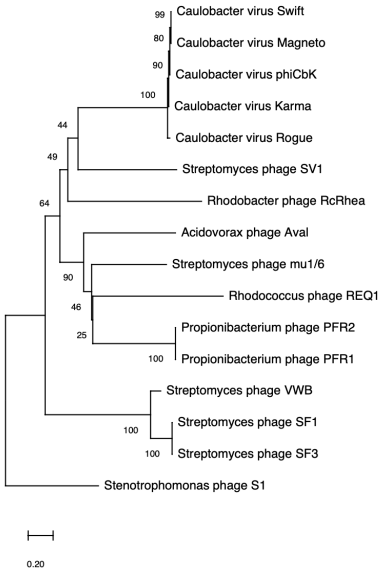

Figure 4.6: Phylogenetic tree of the major capsid proteins of Aval and related phages, by neighbor-joining method. Statistical support for the internal nodes was determined by 1,000 bootstrap replicates. The scale bar shows 0.2 amino acid substitutions per site. Generated in MegaX.

This analysis reveals the same three clusters within the related phage genomes also verified in the heatmap (Fig 4.5). In contrast with the proteomic tree (Fig 4.4), the result of this analysis show that Aval is more distant to the *Caulobacter* phages than, for instance, the *Propionibacterium* phages PFR1 and PFR2, which are two temperate phages [229]. Similar to the previous analyses, Aval is not included in any specific cluster, which reinforces its genomic differences to any known phage, and its representation as orphan phage of a novel genus.

The genome was annotated using Patric [212] and manually curated, predicting a total of 48 open reading frames (ORFs) on both strands. The functions of 22 proteins encoded by the distinguished ORFs could be predicted on the basis of their similarity to known proteins, by manual verification with Blastp analysis. Features of all the genes, including their position and orientation, the sizes of the encoded proteins, and their closest known homologs can be found in Table B.1 in Appendix B. The genome organization is visualized in Figure 4.7.

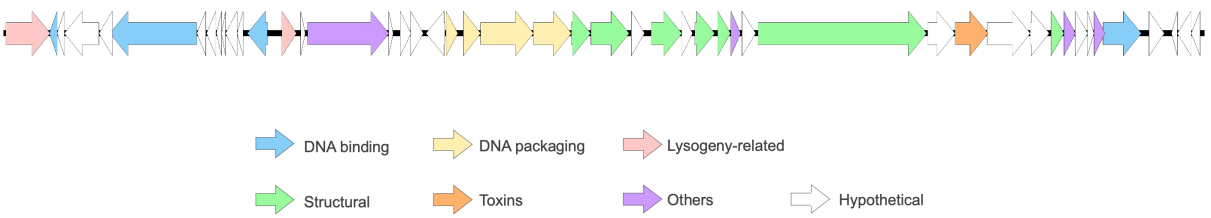

Figure 4.7: Genome map of phage Aval. The arrows indicate predicted ORFs and the direction of their translation: in white—encoding hypothetical proteins, blue-encoding DNA associated proteins, green-encoding structural proteins, yellow—encoding DNA packaging proteins, pink-encoding lysogeny related proteins and purple-encoding other proteins. Generated with EasyFig.

The first module, comprised of DNA-associated genes, starts with an integrase gene (gp1), that is commonly found to mediate the integration of the phage genome on its host by efficient site-specific recombination [230]. In gp6, a chromosome/plasmid partitioning protein, ParB, is encoded. Partitioning systems enable the segregation of plasmid molecules into both daughter cells at division, and has been

described as a factor in the stable maintenance of temperate phages that replicate as extrachromosomal prophages [231]. In this module can also be distinguished different genes encoding transcriptional regulators/regulatory proteins, such as the AlpA family phage regulatory protein (gp2), a helix-turn-helix (HTH) domain-containing protein (gp13) and the transcriptional activator CII (gp14). AlpA acts as a transcriptional regulator of the slpA gene whose activation is necessary to suppress two phenotypes- the overproduction of capsular polysaccharide and the sensitivity to UV light [232]. The HTH domains play a role in DNA repair and replication, RNA metabolism and protein–protein interactions [233]. The HTH encoded by gp13 is a Cro/C1-type conserved domain involved in the DNA-binding of the phage repressor protein C (COG2932), a repressor that is linked to phage lysogenic development. The transcriptional activator CII, encoded by gp14, is also associated with a temperate lifestyle. CII is responsible for the transcription of gene products, such as integrase genes, required for the establishment of a latent state, playing a key role in determining whether the bacteriophage will incorporate its genome or follow the lytic cycle [234].

In the end of this module, gp21 encodes a small nucleic acid-binding protein, His-Asn-His (HNH) endonuclease, right before the small and large terminase subunits, encoded by gp22 and gp23 respectively, all representing key components in phage DNA packaging [235]. This packaging module also harbors gp24, encoding the phage portal protein, which plays a crucial role by forming a portal to DNA passage during packaging and ejection. This protein is also involved in the formation of a junction between the phage head and the tail proteins, bridging to the structural module, represented in green in the Figure 4.7. The structural module, from gp24 to gp39, begins with the genes involved in the head/capsid structure and assembly- encoding the mentioned phage portal protein (gp24), a prohead protease (gp25) and the major capsid protein (gp26), followed by gp28, gp30 and gp31 that encode tail components, and gp32 encoding the tail length tape-measure protein.

It was also found a pyocin knob domain-containing protein, encoded by gp36, and a DNA adenine methylase encoded by gp44. A pyocin is a phage tail-like bacteriocin that employ the same contractility to kill competing bacteria by dissipating their membrane potential. DNA methyltransferases catalyze the transfer of methyl groups to DNA and are present in the genomes of various lytic and lysogenic phages [236].

4.2.2 Protein Isolation

To complement the genome annotation, structural phage proteins were first extracted from purified virions and then analyzed on an SDS-PAGE gel (Figure 4.8). Each band was assigned a specific identity based on the molecular weight of the predicted protein sequences.

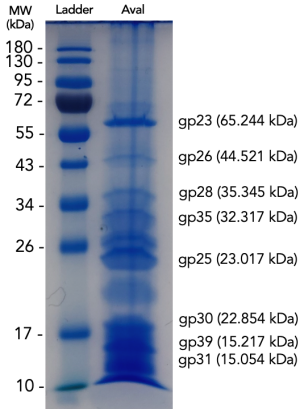

Figure 4.8: Analysis of the AvaI structural proteins by SDS-PAGE (12%). On the right side of the figure are indicated the proteins predicted to correspond to each band, based on the known molecular weight.

The band at the highest molecular weight, around 65 kDa, is likely to correspond to the terminase large subunit, encoded by gp23. The second (44 kDa) matches the predicted size of gp26 that encodes the major capsid protein, followed by a band at 35 kDa that presumably coincides with the gp28-encoded HK97 family protein, that is a putative tail component. Next, a band at 32 kDa matches the size of gp35, a protein with no annotated function. Since this protein is also positioned close to structural cassette, it is then hypothesized that this hypothetical protein has a structural function. A band at 24 kDa can correspond to gp25, a HK97 family prohead protease and the following band at around 19 kDa can possibly be associated with gp30 that encodes a phage tail protein. The bands at molecular weights lower than 17 kDa do not have a clear separation in the gel, not being easily distinguished. Nevertheless, it is possible to see at least one band around 15 kDa with two probable matches, the structural protein P5 (gp39) and a phage tail assembly chaperone (gp31).

It should be noted that mass spectrometry analyses would be required to confirm the identity of the resolved proteins.

4.2.3 Infection Curve

In order to investigate the infection profile of the temperate phage Aval, a killing curve was performed in GBBC 3357 with MOI 0.1, presented in Figure 4.9.

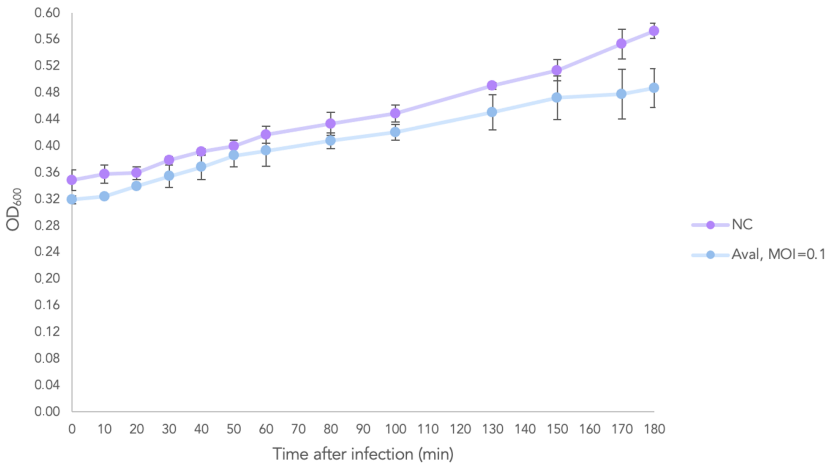

Figure 4.9: Infection curves of *A.valerianellae* GBBC 3357 with Aval with MOI 0.1. The change in optical density (OD_{600}) during growth of *A.valerianellae* GBBC 3357 with the presence of phage is followed through time. The negative control is indicated with lilac • and Aval (MOI 0.1) with blue •. Error bars indicate the standard deviation and are based on three independent repeats.

It is observed that the evolution of the optical density through time of the negative control appear to follow the same profile of the infected culture for the first 100 minutes. After that, a slight difference is noticed, in which the optical density of the negative control has a higher increase than the infected culture. This shows that the infection with Aval, with a MOI of 0.1, only has a minor impact on the host's growth.

4.2.4 Assessing the Lysogen Activity of Aval

Indicators associated with lysogenic behavior were found in Aval, including the formation of turbid plaques and the difficulty to amplify in high concentrations, including the lysis activity in liquid medium and the presence of specific genes. More specifically, the presence of an integrase gene is usually used as another hallmark for lysogenic activity, even if it may not be a definite confirmation of the ability

to lysogenize a host [208]. Therefore, a phenotypic test was used to screen for lysogen activity in Aval, relying on the identification of spontaneous phage release from their lysogenized host and gain of resistance.

This assay was performed in two different hosts: a strain that can be infected by all the three *A. valerianellae* phages - GBBC 3357 - and a strain that can only be infected by Aval - GBBC 3043. After isolating the possible lysogens from turbid plaques (cells that were growing in the presence of the phage Aval), and growing in liquid culture, two spot assays were done to screen for the release of phages. The Figures 4.10 and 4.11 show the results of the strain GBBC 3357. In a first assay the lysogen cultures were spotted on the host lawn (Figure 4.10A), and in a second assay were spotted the supernatants of the same cultures after centrifugation (Figure 4.10B). In this case, lysis spots were observed in both assays in three of the cultures (marked as 1, 2 and 3 in the Figure), revealing spontaneous phage release. With the host GBBC 3043, seven out of nine cultures also showed phage release (images not shown).

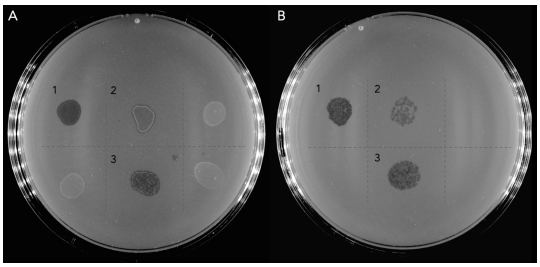

Figure 4.10: Screening of spontaneous phage release from spot assays. A) Spots of the cultures of the possible lysogens; B) Spots of the supernatant after centrifuging the cultures. In 1, 2 and 3 are observed lysis zones.

The cultures that were positive were further used to explore the hypothesis of resistance to the same phage (homoimmunity) with Aval, and in the case of GBBC 3357 also to other phages (superinfection exclusion), using Alfacinha1 and Alfacinha3. For this purpose, the phages were spotted on the lysogens' lawns and also on the wildtype host lawn to compare. As observed in Figure 4.11, the lysogens did not acquire complete resistance to Aval, Alfacinha1 and Alfacinha3. However, it is possible to see an impact on the susceptibility of the three GBBC 3357 lysogens to Aval since there is approximately a 10-fold difference in the production of plaques when comparing to the wildtype host (more noticeable in the lower concentration spots of phage (10^2 - 10^3 PFU/mL)). In GBBC 3043, all the lysogens showed resistance to Aval (images not shown).

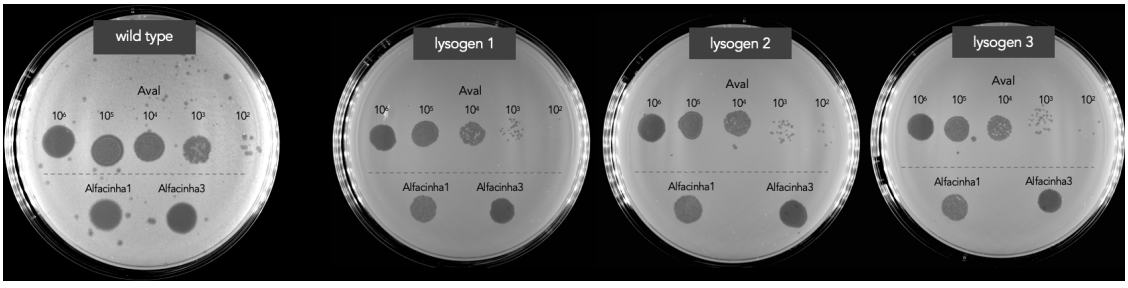

Figure 4.11: Screening of homoimmunity and superinfection exclusion in three GBBC 3357 lysogenized with Aval. Aval was spotted in a sequential dilution in the wild type host and in the three possible lysogens, along with Alfacinha1 and Alfacinha3 with a single spot.

Once it was verified spontaneous release of phages in the first two assays, and a decrease in the susceptibility to Aval (homoimmunity), this assay confirmed that Aval can indeed adopt a temperate lifestyle as suspected.

4.2.5 Discussion

This section described the genomic characterization of Aval, one of the first isolated bacteriophages infecting *A. valerianellae*. The difficulties presented in amplifying this phage at high concentrations and the inconsistent production of both clear and turbid small plaques did not allow a further laboratory microbiological characterization, discarding Aval as a biocontrol agent. Despite this, a deep genomic analysis of this phage, along with a phylogenetic analysis, offered new and interesting findings, revealing that it is a representative of a novel genus and the association with a temperate lifestyle. Besides the genomic indicators, the lysogenic activity of Aval was also experimentally confirmed with a phenotypic test in two different hosts.

Intriguingly, Aval showed to have a highly specific and narrow host range, able to lyse only three out of nineteen strains of *Acidovorax valerianellae*. However, the host range analysis was only based on the successful production of plaques. In the case of a temperate phage, it can also integrate the host's genome after infection without killing the bacteria. Thus, Aval might potentially be infecting other hosts without that being detected. On the other hand, just as there is the possibility of Aval to integrate into the genome, there may also be another prophage already integrated in the same host. As there is still no information about the genome of the strains in collection, it can be possible that they contain integrated prophages, such as previously reported for *A. citrulli*, in which at least seven integrated prophages were found in the genome [115]. Prophages are known to cause autoimmunity, making the strain that is parasitized by a specific phage resistant to a superinfection by other phages, including themselves (homoimmunity) [121]. This phenomenon is also referred to as superinfection exclusion and can be achieved through a wide variety of mechanisms, mainly involving alterations to the cell surface or other cell envelope components that block phage receptors [237] or prevent DNA entry into the cytoplasm [122]. Thus, the presence of integrated prophages that inhibit Aval superinfection could also be a hypothesis to its narrow host range. Also Aval showed ability to cause homoimmunity, as experimentally demonstrated in the phenotypic test performed in two different *A. valerianellae* strains. The presence of integrated prophages could be, for instance, verified by inducing them into the lytic cycle, for example with the use of mitomycin C. Indeed, several prophages were already discovered and isolated after exposure with mitomycin C, such as the *Propionibacterium* phages PFR1 and PFR2 [229].

TEM images of Aval revealed a typical myovirus phage with an icosahedral head and a long contractile tail. It was also observed in both phages of the image (Fig 4.1C) that the tail sheath was contracted, and the capsid already empty, indicating a previous ejection of the DNA, and so, the presence of several inactive phages in the stock. That suggests that the stock, after the removal of the bacterial cells by filtration, or even after PEG purification, still contain molecules that act as receptors to the phages, allowing them to bind and eject the DNA but without producing any progeny. Indeed, PEG precipitation has been shown to not completely remove endotoxins [238, 239], typically requiring downstream implementation of other endotoxin removal techniques, such as LPS affinity interactions, two-phase extractions, ultrafiltration, affinity chromatography or anion exchange chromatography [240]. Endotoxins, also known as lipopolysaccharides (LPS), are found in the outer membrane of Gram-negative bacteria and have been reported to commonly act as phage receptors [105]. For instance, LPS was shown to be the receptor of the *Pseudomonas* phage JG004 [241]. Therefore, problems faced in Aval amplifications can rely not only in the possibility of lysogenic behavior, but also because of a relatively fast inactivation of the phages in the stock due to interaction with possible host receptors.

Surprisingly, although the TEM images showed a *Myoviridae* morphology, the genomic features all pointed to a *Siphoviridae* classification. Indeed, the known phages with higher similarity with Aval, presented in the proteomic tree (Fig 4.4), all belong to the *Siphoviridae* family. Also, its large terminase subunit clustered together with the *Siphoviridae* member *Escherichia* phage HK97 (Fig 4.3) and some of

the annotated structural proteins, such as the tail component (gp28) and the prohead protease (gp25), are genomically associated again with the HK97 family. These inconsistencies between morphology and genome-based relationships with other tailed phages support a system of taxonomical classification not based on the three classical families *Myoviridae*, *Podoviridae*, and *Siphoviridae*, such as ICTV proposed recently [101], by replacing them with monophyletic, genome-based families.

Different approaches were used for providing further insight into the diversity and taxonomy of Aval. The proteomic tree (Fig 4.4), the heatmap (Fig 4.5) and the phylogenetic analysis (Fig 4.6) demonstrate that Aval is a novel phage without any relevant homology with any known phage- an orphan phage- and so, a new genus should be proposed, e.g. 'Avalvirus' genus. The genome of Aval showed an organization of three main modules (Fig 4.7), beginning with DNA-binding, followed by DNA packaging and structural modules. In the first module, genes related with lysogenic activity were identified, namely encoding the transcriptional activator CII, that determines if the phage will incorporate the genome or follow the lytic cycle, and the integrase that mediates the incorporation in the host's genome, upon activation by CII [234]. In the same module, a partitioning protein was found, that shows an alternative ability to maintain a temperate lifestyle, by replication as an extrachromosomal prophage [231]. It is interesting to notice the simultaneous presence of these two different ways of prophage maintenance in Aval's genome, since genes with partitioning functions are usually present in temperate phages as a replacement of the integration cassette [231]. Using third generation sequencing technologies (Oxford nanopore sequencing), the behavior of Aval inside the bacterial cell could be captured. More specifically, using long read sequencing, the flanking regions of the phage genome could be captured, revealing the genomic integration of the phage into the bacterial genome, or its extrachromosomal state [242]. Additionally, the encoded DNA methyltransferase might be complementing this 'double ability' to generate and maintain stable lysogens by protecting the phage from host's restriction endonucleases, as has been shown in various lytic and lysogenic phages [236]. If, on the one hand, the phage tries to protect itself from its own host, on the other hand it is also conferring it a competitive advantage by turning the host more virulent through lysogenic conversion. Indeed, several encoded bacteriocins, such as the pyocin, have been discovered in temperate phages, providing evolutionary benefits to the infected host in intraspecies competition [243], once they can be deployed to kill the bacterial neighbors.

In conclusion, genomic and experimental evidence revealed Aval as a novel and orphan phage that can adopt a temperate lifestyle. In addition, Aval is likely associated with lysogenic conversion, as it encodes bacteriocins that can provide the host an evolutionary advantage over competitors.

4.3 New Clade Consisting of two Phage Genera Infecting *A. valerianellae* and *A. cattleyae*

4.3.1 Genomic Analysis

In order to characterize the other three isolated bacteriophages Alfacinha1, Alfacinha3 and Acica, whole genome sequencing was performed. The main genomic characteristics determined for these three phages are summarized in Table 4.2.

No accentuated differences could be detected in the reads' coverage over the assembled genome. The packaging strategy was then identified by comparing the large terminase protein sequences with those of phages with known packaging mechanisms in a neighbor-joining tree (Figure 4.12), following the description of Merrill and colleagues [227]. The large terminase sequences of Acica, Alfacinha1 and Alfacinha3, clustered together with the ones from the *Pseudomonas* virus phiCTX and *Escherichia* phages 186 and P2, that present 5' *cos* ends [227]. The physical start of the phages was then deter-

mined by aligning their genomes against the one from *Escherichia* phage P2.

Table 4.2: Summary of the main bioinformatic characteristics of Alfacinha1, Alfacinha3 and Acica.

	Genome length (bp)	GC content (%)	# ORFs	# Annotated functions
Alfacinha1	40,274	65.2	53	33
Alfacinha3	40,526	65.0	53	33
Acica	37,472	67.5	49	32

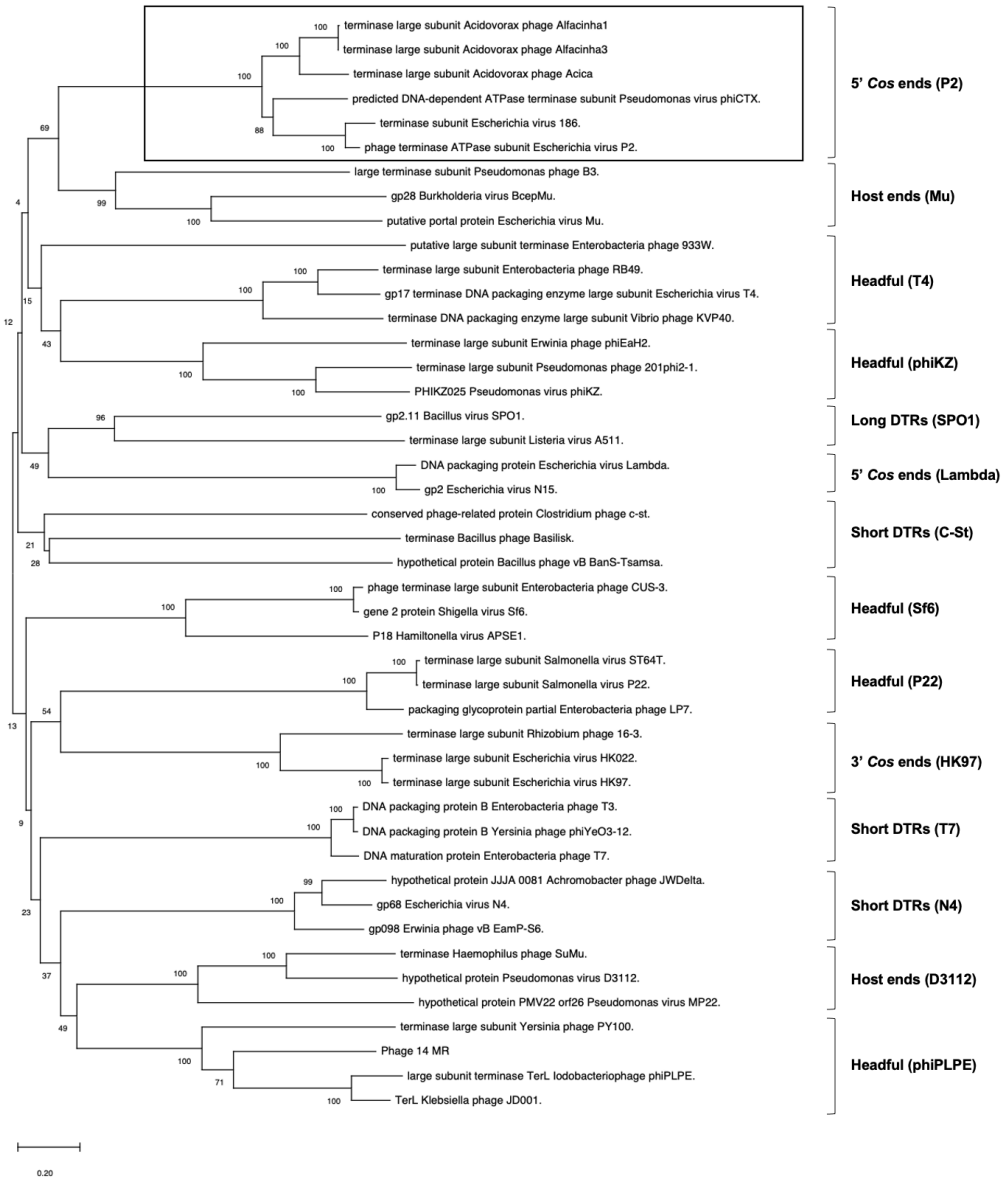

Figure 4.12: Neighbor-joining tree of large terminase protein sequences of Alfacinha1, Alfacinha3, Acica and phages whose packaging strategy is known. The black rectangle indicates Alfacinha's and Acica's cluster. The brackets indicate the packaging strategies experimentally determined for each cluster, according to Merrill et al. [227]. Bootstrap values are for 1000 trials. The scale bar shows 0.2 amino acid substitutions per site. Generated in MegaX.

The viral taxonomy of the three bacteriophages and their similarity with other known phages was investigated by generating a proteomic tree based on genome-wide sequence similarities (Figure 4.13), using the ViPTree server [221]. VIRIDIC [222] was then used to compute the pairwise intergenomic

similarities amongst the related viral genomes present in the heatmap of the Figure 4.14.

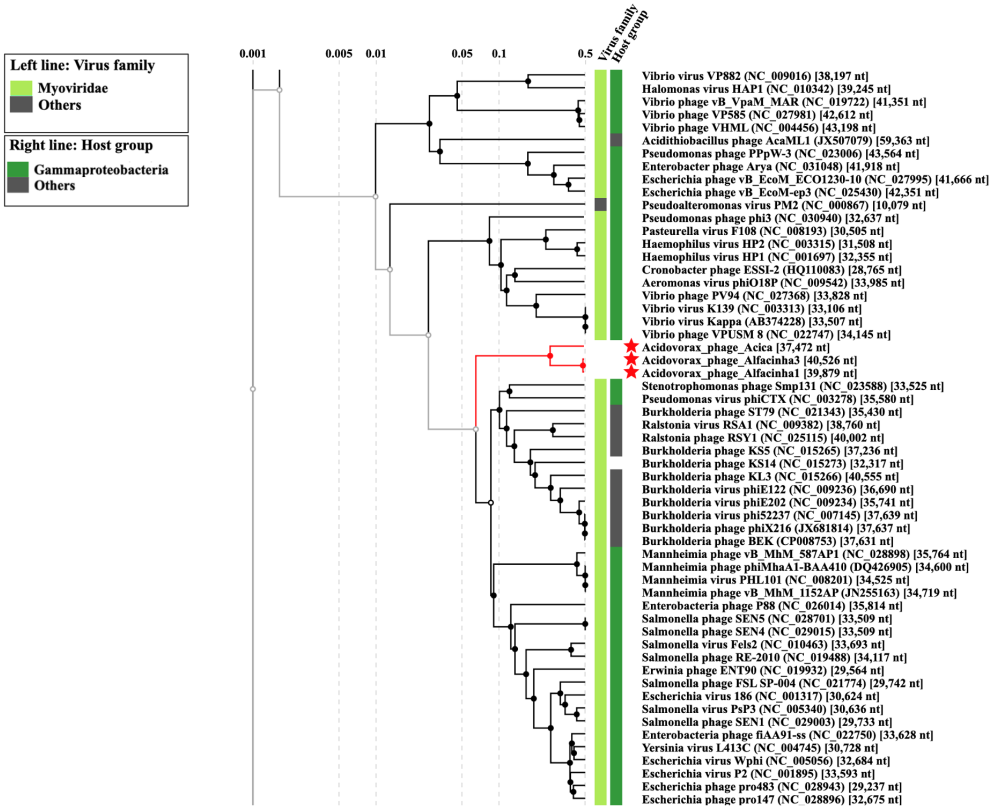

Figure 4.13: Proteomic tree based on global genomic similarity relationships between Acica, Alfacinha1, Alfacinha3 and other known phages, predicting the virus family and host group. Only the section with the most related phages of the tree is presented in this figure. Alfacinha1, Alfacinha3 and Acica are indicated with red stars. Generated in ViPTree server.

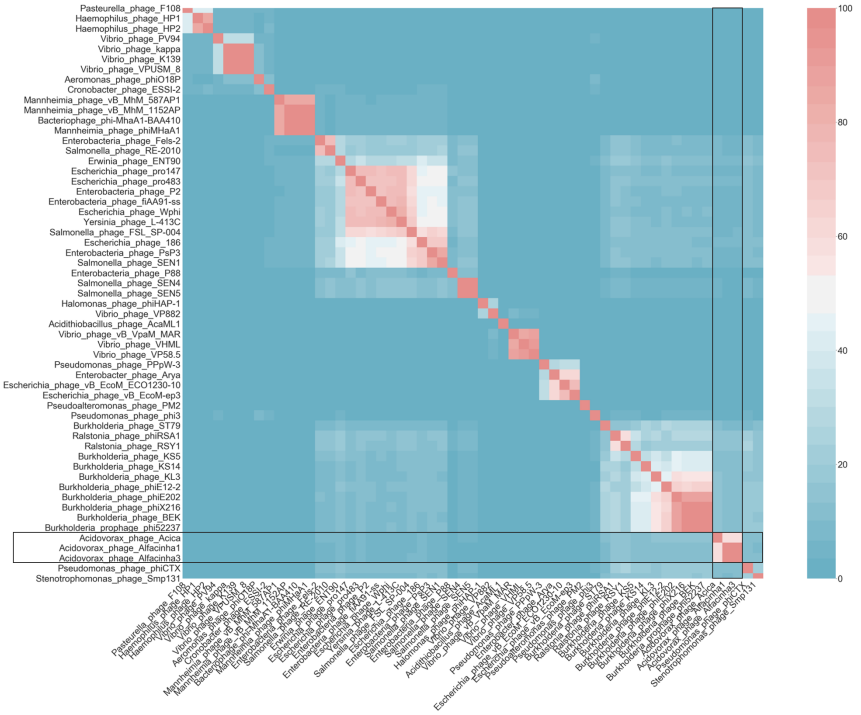

Figure 4.14: Heatmap based on the calculated sequence similarities (using VIRIDIC) between Alfacinha1, Alfacinha3, Acica and their most related phages.

The presented section of the proteomic tree (Figure 4.13) shows that Alfacinha1, Alfacinha3 and Acica clustered together with phages of the *Myoviridae* family, that is in accordance with the morphology shown in the TEM images (Figure 4.1). It is observed that these three phages represent a single cluster, related with two other clusters, one mainly composed of *Burkholderia* phages, and the other of *Mannheimia*, *Salmonella* and *Escherichia* phages, mainly belonging to the *Peduovirinae* subfamily. Within the cluster, Alfacinha1 and Alfacinha3 are more closely related to each other than Acica. This is also confirmed by the heatmap, in Figure 4.14, in which is possible to distinguish more quantitatively the relationships within and between the different clusters. Indeed, while Alfacinha1 and Alfacinha3 have a similarity of 97.5%, belonging to the same genus and species, Acica is only around 57% similar to them, thus belonging to a different genus, based on the current ICTV guidelines [228]. These three phages show homologies of less than 15% to all the remaining phages, with a maximum similarity of around 14% with the *Ralstonia* phages phiRSA1 and RSY1. Therefore, two novel phage genera, one composed by Alfacinha1 and Alfacinha3 and the other composed by Acica, were found.

The sequences of highly conserved proteins were used in phylogenetic analyses in order to explore possible evolutionary relationships among the most related phages. Figure 4.15 shows the neighbor-joining trees of the major capsid protein and the large terminase subunit of the new *Acidovorax* phages Alfacinha1, Alfacinha3 and Acica and related phages.

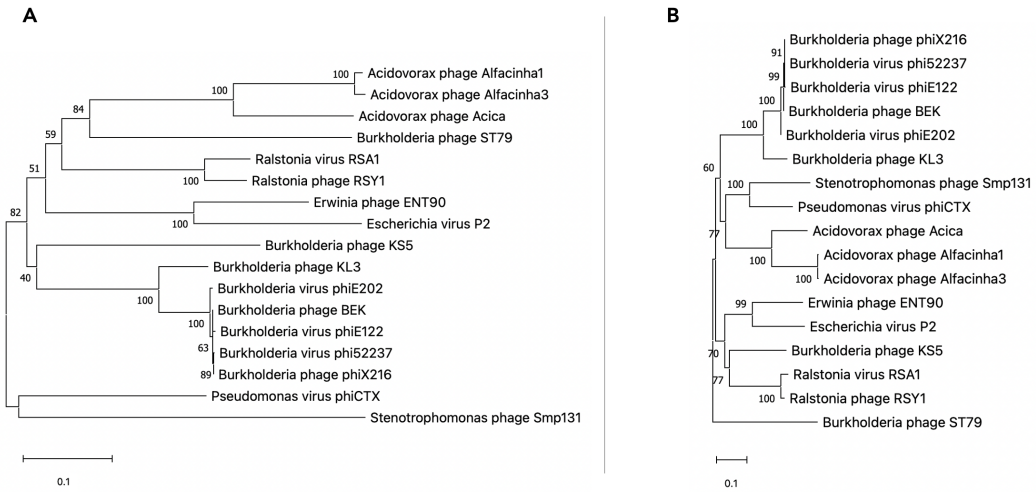

Figure 4.15: Phylogenetic trees of the major capsid protein (A) and large terminase subunit (B) of Alfacinha1, Alfacinha3, Acica and related phages, by neighbor-joining method. Statistical support for the internal nodes was determined by 1,000 bootstrap replicates. The scale bar represents 0.1 amino acid substitutions per site. Generated in MegaX.

In both phylogenetic trees the proteins of the *Acidovorax* phages stayed in an isolated cluster. In the case of the major capsid protein (Fig 4.15A), they are closer to the *Burkholderia* phage ST79 protein, while the large terminase subunits (Fig 4.15B) are more related with the ones from *Pseudomonas* phage phiCTX (as also shown in Fig 4.12) and from *Stenotrophomonas* phage Smp131. Interestingly, it is clear the difference between the phylogenetic relationships of the major capsid and large terminase, since the phage with the most closely related major capsid is, on the other hand, one of the phages with the most distant large terminase, and vice versa. It should also be noted that, although the *Ralstonia* phages were shown to be the most genomically similar to the *Acidovorax* phages, their proteins, especially the large terminase, are phylogenetically more distant.

To better understand the genome organization of these three phages and to compare it with other known related phages, their genomes were annotated using Patric [212] and manually curated. The encoded ORFs, ranging from 49 to 53 (Table 4.2) were identified, and some functions could be assigned

by verifying similarity at the protein level by Blastp analysis. Features of all the genes, including their position and orientation, the sizes of the encoded proteins, and their closest known homologs can be found in Tables B.2, B.3 and B.4 in the Appendix B. Alfacinha1, Alfacinha3 and Acica genome maps and their comparison to the genomes of the *Ralstonia* phages phiRSA1 (accession number: NC_009382.1) and RSY1 (accession number: NC_025115.1) are visualized in Figure 4.16.

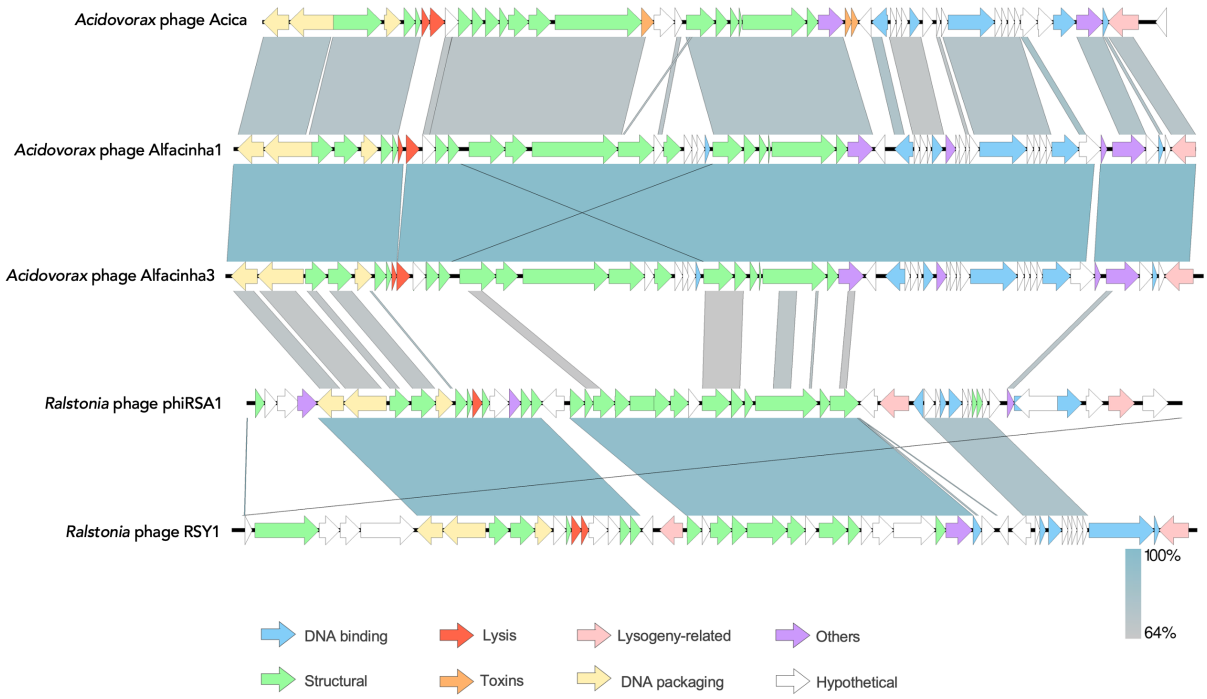

Figure 4.16: Genome map of the *Acidovorax* phages Acica, Alfacinha1 and Alfacinha3 and of the *Ralstonia* phages phiRSA1 and RSY1. The arrows indicate predicted ORFs and the direction of their translation: in white—encoding hypothetical proteins, blue-encoding DNA associated proteins, green-encoding structural proteins, red-encoding lysis related proteins, orange-encoding toxins, yellow—encoding DNA packaging proteins, pink-encoding lysogeny related proteins and purple-encoding other proteins. Generated with EasyFig.

All the genomes appeared to be similarly structured, starting with a DNA packaging module, followed by lysis-related genes, the structural module and finally a module encoding DNA-binding proteins.

The initial module is nearly identical in all the five phages, harboring genes required for DNA packaging and also for the capsid synthesis. In Alfacinha and Acica phages, gp1 and gp2 correspond to the packaging-related genes of the portal protein and the large subunit of the terminase, respectively. In Alfacinha phages, these are followed by gp3 and gp4, encoding a capsid scaffolding protein and the major capsid protein (gp3 in Acica), respectively, and ending with the gp5 gene of the terminase small subunit and gp6 for the head completion protein.

Just before the structural module, a region of lysis-associated genes is present. All the five phages have genes encoding for holins, small membrane proteins that allow bacteriolytic enzymes to escape to the periplasm and to attack the cell wall. Additionally, the *Acidovorax* phages have genes encoding a lysozyme, in the case of Alfacinha1 and Alfacinha3, and an endolysin, in the case of Acica. These enzymes are able to degrade the peptidoglycan layer of the host's cell wall from within, resulting in cell lysis and the release of phage progeny [244].

The structure module that follows is mainly comprised of genes encoding proteins associated with the tail and baseplate structure and assembly. In the case of Acica, nine out of fifteen genes encode tail proteins (gp6, gp10, gp15, gp20-gp25) and the genes gp12, gp13 and gp14 encode baseplate proteins. Alfacinha1 and Alfacinha3 have ten genes encoding tail proteins but only one encoding a baseplate protein (gp13).

The structural module is followed by a module of genes encoding DNA-binding proteins, including transcriptional regulators, such as a helix-turn-helix transcriptional regulator (encoded by gp22 in both Alfacinha phages and by gp30c in Acica), an Ogr/Delta-like zinc finger family protein (encoded by gp35 in both Alfacinha phages and by gp34 in Acica) and an AlpA family phage regulatory protein (encoded by gp51 in both Alfacinha phages and by gp47 in Acica). There are also genes encoding DNA modifying proteins, such as the phage replication endonuclease, responsible for the initial scission required for the linearization of the circular replication intermediates at the cos-sites during packaging of the phage DNA [245], and a cytosine-specific methyltransferase that can catalyze the transfer of methyl groups to DNA. Notably, the Blastn comparison of this module does not show any similarity between the *Acidovorax* and the *Ralstonia* phages. In the end of the genome, all the five phages present a site-specific integrase, that is often related with integration of the phage genome into the bacterial chromosome.

In contrast with the other phages, Acica's genome showed the presence of toxin-related genes. The BrnT/BrnA toxin-antitoxin system consists of a set of two closely linked genes (gp27 and gp28) that, when together, form a high-affinity, neutral complex that encode both a stable toxin protein and the corresponding antitoxin. A rearrangement hotspot (RHS) repeat, encoded by gp17, is also present, which was previously reported to mediate intercellular competition by inhibiting the growth of neighboring cells [246].

To further explore and compare the differences between the new isolated *Acidovorax* phages, a proteome comparison was performed, and can be visualized in Figure 4.17.

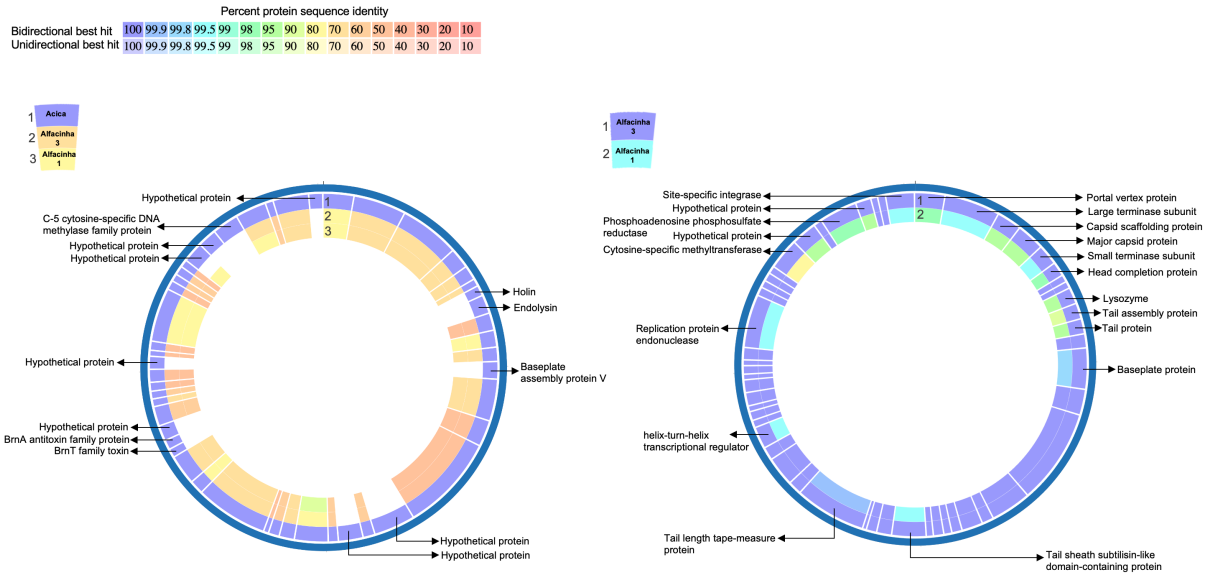

Figure 4.17: Proteome comparison of Alfacinha1, Alfacinha3 and Acica. The proteomic comparison between the phage Acica (outer circumference) and the phages Alfacinha1 (inner circumference) and Alfacinha3 (middle circumference) is shown on the left side and between Alfacinha1 (inner circumference) and Alfacinha3 (outer circumference) on the right side. The colours indicate the percentage of protein sequence identity in the subject genome to the matching protein sequence in the reference genome. A white space indicates that a gene/protein is missing compared to the reference.

On the circle on the left side of Figure 4.17, it is shown the comparison between the Acica's genome, represented by the purple outer circumference, and the genomes of Alfacinha1 and Alfacinha3. The sequence similarity found between Acica's proteins and the ones from the other phages ranged from 51%, found in a baseplate protein, and 90% in a protein of the tail sheath. However, there are also proteins for which no similarity was found (represented by the gaps in Figure 4.17), including the lysis proteins, the baseplate assembly protein, the toxin-antitoxin system, the DNA methylase and seven proteins with unknown function.

Previously, the two Alfacinha phages showed to be very similar, sharing an intergenomic similarity of 97.5% and an identical genome organization, as seen in Figure 4.16. On the circle of the right side of the Figure 4.17 it is possible to evaluate their differences. Part of the genome, from gp12 to gp45, appears to be highly conserved (with more than 99.5% of similarity) while the remaining part is more variable. The latter is mainly comprised of proteins associated with DNA packaging, such as the portal protein and the terminase subunits, and with capsid synthesis, including the capsid scaffolding protein, the major capsid and the head completion/stabilization protein. Also the lysozyme and some of the tail and baseplate proteins showed less similarity. Additionally, differences were found in DNA-associated proteins, such as the helix-turn-helix transcriptional regulator, the endonuclease, the cytosine-specific methyltransferase and the integrase.

4.3.2 Protein Isolation

As was done for the phage AvaI, the structural proteins of Alfacinha1 were analyzed on an SDS-PAGE gel, after their extraction from purified virions. The resulting gel is shown in Figure 4.18, where each band was assigned a specific identity based on the molecular weight of the predicted protein sequences. It is hypothesized that, given the high similarity found between the proteins of Alfacinha1 and Alfacinha3, the band profile of Alfacinha3 would be equivalent to the one of Alfacinha1.

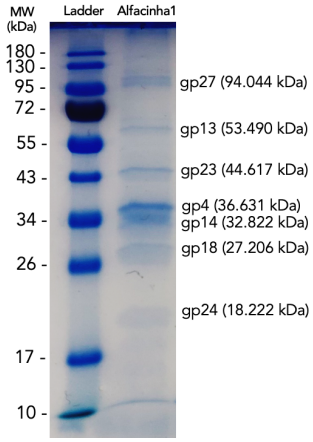

Figure 4.18: Analysis of Alfacinha1 structural proteins by SDS-PAGE (12%). On the right side of the figure are indicated the proteins predicted to correspond to each band, based on the known molecular weight.

The bands at 95 kDa and 56 kDa probably correspond to the tail length tape-measure protein and to the baseplate protein, encoded by gp27 and gp13, respectively. The third (44 kDa) matches the predicted size of gp23, that encodes a protein of the tail sheath. Two bands at molecular weight about 34 kDa follow, that presumably coincide with the proteins encoded by gp24 and gp14, the major capsid protein and a tail protein, respectively. Next, a band at 27 kDa matches the size of the envelope protein (encoded by gp18). The last band at around 19 kDa may correspond to the gp24-encoded major tail tube protein. It is noted that to confirm the identity of the resolved proteins, mass spectrometry analyses would be required. Nevertheless, the profile observed in the gel appears to be in accordance with the structural proteins predicted by the genomic analysis.

4.3.3 Microbiological Characterization of Alfacinha3

From the four isolated *Acidovorax* phages, Alfacinha3 was the only one that could easily reach high titers (10^{11} PFU/mL), completely lysing the bacterial cultures. It also has a high specificity for *Acidovorax*

valerianellae strains, unlike Alfacinha1 which can infect *A. cattleyae*. Since it produces clear plaques and does not show the presence of genes encoding toxins or related to lysogenic conversion, it was chosen for further microbiological experiments and bioassays in order to access its potential in biocontrol.

4.3.3.1 Adsorption Assay

The phage infection process starts with adsorption to the bacterial receptor. An adsorption assay (Figure 4.19) was performed with Alfacinha3 at MOI of 0.01 to assess the speed of irreversible adsorption of the phage particle to the host cell (*A. valerianellae* GBBC 3161) and the efficacy of this process.

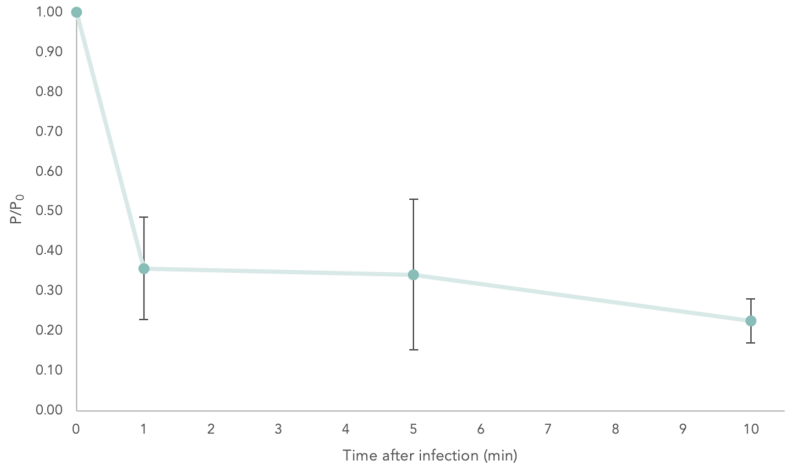

Figure 4.19: Adsorption curve of Alfacinha3 to the host strain *A.valerianellae* GBBC 3161, with MOI=0.01. The ratio of non-adsorbed phages (P) to the initial titer (P_0) is followed through time. Error bars indicate the standard deviation and are based on three independent repeats.

After one minute, 64.3% of the phage particles were adsorbed to the host cell and after ten minutes more than 77.3%. Under these circumstances, this gives an adsorption constant, described by Equation 4.1 [247], with B representing the bacterial titer, t the time, P_0 the initial phage titer and P the phage titer after time t, of 3.52 x 10^{-9} mL/min after one minute.

$$k = \frac{2.3}{B * t} * \log_{10} \frac{P_0}{P} \tag{4.1}$$

Upon comparison of the adsorption constant to other *Myoviridae* phages, Alfacinha3 adsorption was slower than that of the *Dickeya* phage LIMEstone2 (2.1 x 10^{-8} mL/min) [248] the *Pseudomonas* phage KIL3 (7.5 × 10^{-9} mL/min) [152], but still faster than what was reported for the phage T4 (2.4 × 10^{-9} mL/min) [247]. On the other hand, when comparing the total amount of phages irreversibly adsorbed to the host cell after ten minutes, Alfacinha3 shows lower numbers (77.3%), than, for instance, the phages LIMEstone2 and KIL3, that adsorb more than 99%.

4.3.3.2 Infection Curves

To assess the speed of the infection and cell lysis process, exponentially growing cultures of *A. valerianellae* GBBC 3161 were infected with the phage Alfacinha3 at different MOIs, and the variation in optical density (OD_{600}) during growth was monitored through time (Figure 4.20).

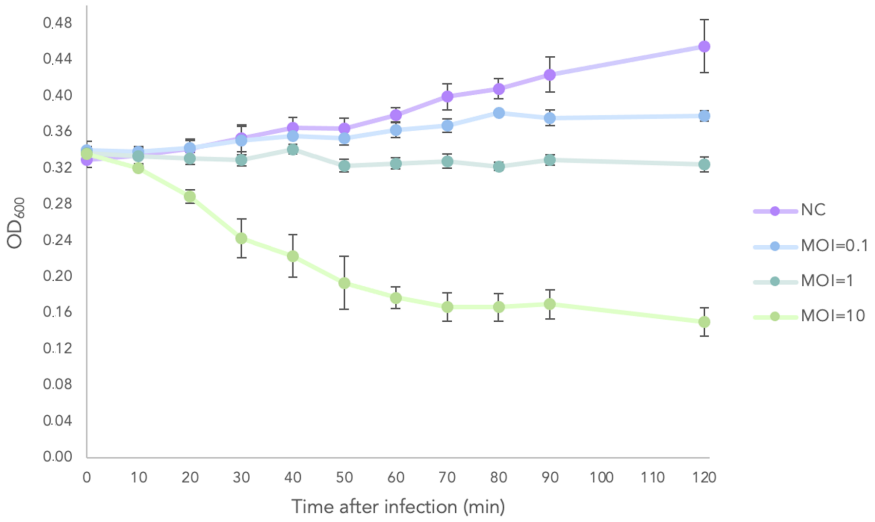

Figure 4.20: Infection curves of *A.valerianellae* GBBC 3161 infected with phage Alfacinha3 at different multiplicities of infection (MOI). The variation in optical density (OD_{600}) during growth of *A.valerianellae* GBBC 3161 with different concentrations of phage is followed through time. The negative control is indicated with lilac (●), MOI 0.1 with blue (●), MOI 1 with turquoise (●) and MOI 10 with light green (●). Error bars indicate the standard deviation and are based on three independent repeats.

When comparing the optical density of the infected bacterial cultures with the negative control, it is observed that after 40 minutes, a decrease in growth is detected with all the MOIs, demonstrating the virulence of the phage. Noteworthy, a steeper decline reaching an OD_{600} of almost 0.1 is observed with MOI 10 within 120 minutes.

4.3.4 Seed Bioassay

Considering that *Acidovorax valerianellae* transmission by contaminated seeds is discussed as a major infection source, the capability of bacteriophages to treat contaminated seeds was tested. To this end, lamb's lettuce seeds were first inoculated with *A. valerianellae* strain GBBC 3161 (10^8 CFU/mL) and then primed with the phage Alfacinha3 (10^9 PFU/mL). The final concentration of phage and bacteria per gram of seeds was determined after phage incubation overnight and the results are present in Figure 4.21.

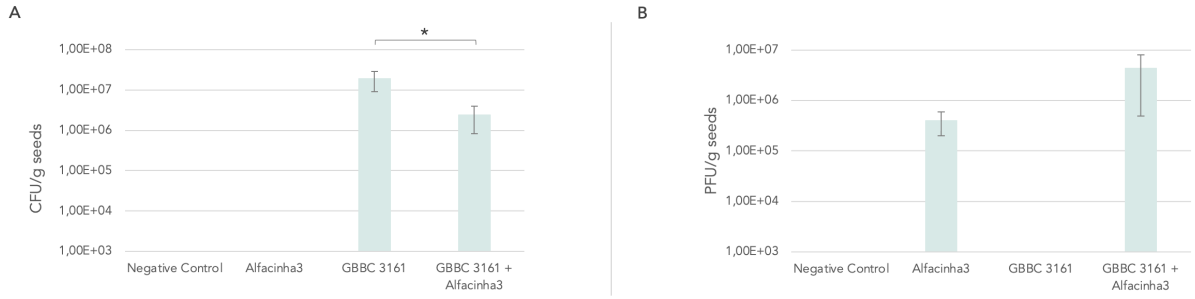

Figure 4.21: Bacterial (A) and phage (B) concentration on the seeds for the four different conditions - Negative control, bacteria only (infected with *A.valerianellae* strain GBBC 3161 at 10^8 CFU/mL), phage only (primed with Alfacinha3 at 10^9 PFU/mL) and bacteria plus phage (GBBC 3161 + Alfacinha3). Error bars indicate the standard deviation and are based on three independent repeats. Statistical support is based on T-test, showing significant difference in (A) ($p<0.05$) and no significant difference in (B) ($p=0.15$).

After artificially infecting the seeds with *A. valerianellae* (10^8 CFU/mL), a bacterial concentration of around 2 x 10^7 CFU/g seeds was detected inside the seeds. When treated with the phage Alfacinha3,

a 10-fold reduction (equivalent to 87% reduction) could be achieved. It is worth highlighting that despite having applied a phage solution with a concentration of 10^9 PFU/mL, the measured phage concentration inside the seeds was of only 4 x 10^5 PFU/g seed. This can mean that the phages are not easily entering the seeds, or that they possibly have a low persistence inside them, reaching slightly higher concentrations when the host is present since they can infect it and generate progeny.

To evaluate the effects of phage Alfacinha3 on plant growth and symptoms in the presence of *A. valerianellae*, the infected seeds primed with phage solution were grown in MS Agar under laboratorial conditions over 22 days. In Figure 4.22 the seedlings are visualized after growing under four different conditions, A- negative control, B- infected with bacteria, C- primed with phage and D- infected with bacteria and primed with phage.

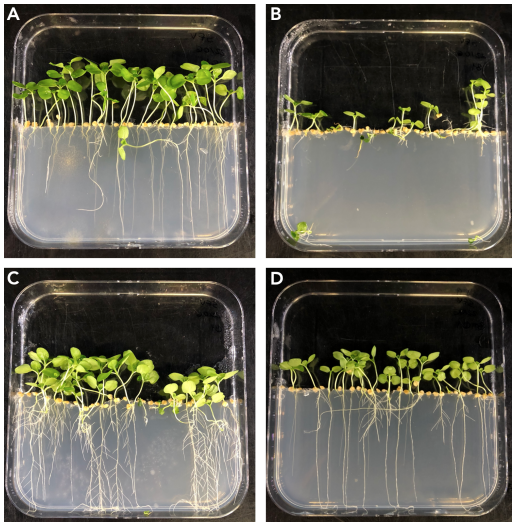

Figure 4.22: Lamb's lettuce seedlings after germination in four different conditions- A) negative control; B) bacteria only (infected with *A.valerianellae* strain GBBC 3161 at 10^8 CFU/mL); C) phage only (primed with Alfacinha3 at 10^9 PFU/mL) and D) bacteria plus phage (GBBC 3161 + Alfacinha3).

A clear difference can be observed between the untreated infected seedlings (B) and the other conditions, showing lower germination and poor growth, with very short roots and small shoots. On other hand, the phage-treated infected seedlings (D) show much higher germination rates and longer roots when comparing with the untreated ones, qualitatively demonstrating a positive effect of the phage treatment on plant growth. Interestingly, the seedlings which were phage-primed appear to have more developed roots, with increased lateral root density and lateral root length, indicating that the phage solution might also be inducing a plant response.

After 22 days of growth, the germination rate, the shoot and root length were measured, and the vigor index was calculated by the product of the shoot and root lengths with the percentage of seed germination. The main parameters are summarized in Table 4.3. The distribution of the shoot (A) and root (B) length data for each condition is presented in the box plots of Figure 4.23.

Table 4.3: Measurements of seedlings grown for 22 days from each condition - Negative control, bacteria only (strain GBBC 3161), phage only (Alfacinha3) and bacteria plus phage (GBBC 3161 + Alfacinha3). The vigor index was calculated by the product of the shoot and root lengths with the percentage of seed germination. The results are based on three independent repeats, each one using 30 seeds per condition. Statistical support is given for the vigor index parameter by the connecting letters ([a] and [b]), based on the comparisons performed with Wilcoxon method (p=0.05).

	Mean shoot length (mm)	Mean root length (mm)	Germination rate (%)	Vigor index
Negative Control	25.4	40.9	96.7	1044.1 [a]
GBBC 3161	9.7	8.5	58.9	62.1 [b]
Alfacinha3	22.5	50.7	90.0	1085.6 [a]
GBBC 3161 + Alfacinha3	20.1	54.2	93.3	1054.3 [a]

The results demonstrate that the addition of the phage Alfacinha3 to the *A. valerianellae* infected seeds increased the germination rate from 58.9% to 93.3% and resulted in a dramatic rise in the vigor index. In addition, the infected seedlings presented a marked reduction in root and shoot length (as also noticed in Figure 4.22). The germination rate of the non-infected phage-primed seeds revealed to be lower than the negative control. On other hand, its vigor index was the highest. This is likely correlated with the root length, since the phage-primed seedlings had longer roots, as observed in Table 4.3 and in the box plots of Figure 4.23B. Also, the phage-treated seedlings revealed longer roots, supporting the hypothesis that there is an interaction between the plants and the phage solution that is causing a phenotype change in the roots.

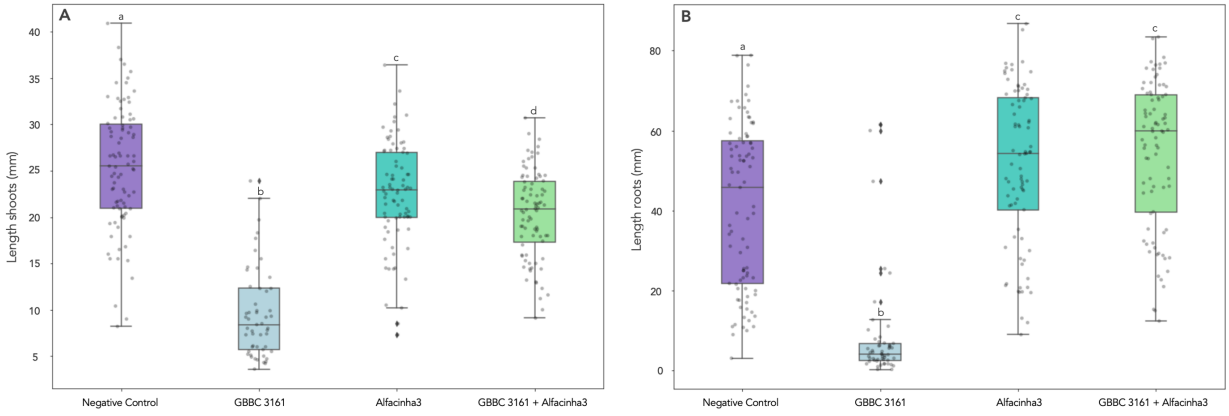

Figure 4.23: Measurements of the shoot and root length after germination in four different conditions - Negative control; bacteria only (infected with *A.valerianellae* strain GBBC 3161 at 10^8 CFU/mL); phage only (primed with Alfacinha3 at 10^9 PFU/mL) and bacteria plus phage (GBBC 3161 + Alfacinha3). The results are based on three independent repeats, each one using 30 seeds per condition. Statistical relevance is represented by the connecting letters based on the nonparametric comparisons performed with Wilcoxon method (p=0.05).

4.3.5 Discussion

This chapter described the characterization of three novel bacteriophages (Alfacinha1, Alfacinha3 and Acica) infecting *Acidovorax valerianellae* and *Acidovorax cattleyae*. Furthermore, the potential use of the phage Alfacinha3 as an agent in biocontrol of *A. valerianellae* was also assessed in a seed bioassay.

Host range analysis demonstrated that the phages infected different strains, covering around 60% of the strain collection. Good candidates for phage biocontrol preferably present a wide host range to maximize the chance of also lysing unknown strains in field conditions. However, it also must be

species specific to avoid detrimental effects to other, beneficial bacteria. Although Alfacinha1 showed the widest host range, it infected strains of both *A. cattleyae* and *A. valerianellae*, therefore not favorable to be used as a biocontrol agent. In sharp contrast, Alfacinha3 showed to be specific and to infect a higher number of *A. valerianellae* strains. Genomic differences can modulate host range at different stages, such as in surface-adhesion, genome replication and host cell lysis. They frequently rely on differences in the phage receptor binding proteins, recognizing, in turn, different host receptors. For example, a spontaneous mutation in a tail fiber protein (replacement of a positively charged lysine by an uncharged asparagine) led to changes in the host range of two related phages, *Pseudomonas* phages PaP1 and JG004 [249]. Furthermore, phages can show different adaptations to evade the host intracellular defense systems, protecting their DNA during replication and transcription, as demonstrated by the *Pseudomonas* phage 201w2-1 [250], or for instance, presenting distinct endolysins that lyse divergent cell-wall structures to release phage progeny [251]. Intriguingly, when looking into the genomic analysis, the phages Alfacinha1 and Alfacinha3 share a high similarity (97.5%) and the same genome organization, not presenting a direct straightforward link to this host range diversity. On other hand, the TEM images revealed some morphological differences, especially in Alfacinha1 tail that, besides being narrower, appeared to have convoluted fibers, in contrast with Alfacinha3. Indeed, also the proteome comparison between these phages showed differences, albeit small, on the protein sequence of some of the tail proteins (gp10, gp11, gp23, and gp27). As described before for PaP1 and JG004, small differences such as the replacement of an aminoacid in a tail protein can, nevertheless, lead to significant changes in host range, by altering the interaction with the receptors. It is also worth noting the differences revealed by the proteome comparison in the DNA methyltransferase and in the lysozyme, since they play a role in anti-defense systems and host lysis respectively, which have been suggested to influence phage host range [251]. Therefore, it can be hypothesized that the differences found in these proteins could be correlated with the differences observed in host range. However further investigation would be required to unravel the exact role of the variation observed.

Genome sequencing confirmed that the three new phages Alfacinha1, Alfacinha3 and Acica are very closely related myoviruses, in accordance with the TEM images. The different approaches used for giving insight into their diversity and taxonomy, such as the proteomic tree and the heatmap, along with the phylogenetic analysis, show that these three phages have low similarity to other phage genomes in the database, with a maximum similarity of about 14% with the *Ralstonia* phages phiRSA1 and RSY1. Therefore, they can be considered as novel phages, which should be placed in a new taxonomic group, belonging to the *Peduovirinae* subfamily, such as the majority of the most related phages. It is worth noting that, with the new taxonomic proposal of ICTV, this subfamily will be reclassified up to family level [101], and so these three phages will likely form a new subfamily. Within their cluster, Alfacinha1 and Alfacinha3 present higher similarity between each other (97.5%) than with Acica (57%). Thus, according to the current ICTV guidelines [228], two new different genera should be proposed, e.g. 'Alfacinhavirus' and 'Acicavirus'.

The genome followed the same organization in the three phages, also corresponding to the one of the *Ralstonia* phages phiRSA1 and RSY1. It is comprised of four main modules beginning with DNA-packaging, followed by a lysis-related region, the structural module and ending with the DNA-associated module. Noteworthy, all the genomes finish with a site-specific integrase gene, which is often related with integration into the host's genome, and thereby, with a lysogenic lifestyle. However, Alfacinha1 and Alfacinha3 consistently produced clear plaques and were able to fully lyse the bacterial cultures, exhibiting evidence of following the lytic cycle. In addition, no other lysogeny-related genes were found, such as repressor genes like CII, which are known to regulate the transcription of integrase genes and other elements necessary for lysogenic activity [234]. Phage integration is known to be a host-specific process, in which the integrase relies on distinct target sequences, such as within conserved tRNA

genes, to act [251]. Thus, phages can only integrate into hosts that contain the correct integration target site. As such, it can be hypothesized that either the Alfacinha phages have lost a key gene needed for lysogenic establishment or the host itself can be limiting their integration through the absence of the integrase-recognizing sites. Sequencing of the host genome could confirm whether a phage is generating or not an integrated prophage, by looking for a match in the bacterial genome corresponding to the full length of the phage [252]. Searching for the prophage integration sites, whose exact sequence matches between the phage and bacterial genomes [252], could also help to evaluate the capacity of the phage to specifically integrate that host, even if it is not following the lysogenic route.

Moreover, the phages Alfacinha1 and Alfacinha3 do not encode toxins or other known proteins associated with virulence or antibiotic resistance. In sharp contrast, the phage Acica revealed the presence of the BrnT/BrnA toxin-antitoxin system (gp27 and gp28), that has been reported to regulate stress adaptation and persistence during antimicrobial treatment [253]. In more detail, in response to various environmental stressors, such as low pH and oxidative stress, transcription of the toxin induces a bacteriostatic condition, in which the cells are still viable but unable to proliferate. This condition can be fully reversed by expression of the cognate antitoxins, thereby protecting the cells from long-term starvation. Additionally, Acica encodes a rearrangement hotspot (RHS) repeat (gp17). Despite the functions of this gene family are not yet well understood, it has been reported to be associated with toxin domains, which can be deployed to inhibit the growth of neighboring cell [246]. These toxin systems present in Acica's genome likely contribute to a strong positive selection of its host, thus providing it a competitive and evolutionary advantage. Therefore, even though Acica was found to specifically infect most of the *A. cattleyae* strains collection, its capability to turn its host into a more virulent and competitive pathogen discarded Acica as biocontrol agent. Alfacinha3, on other hand, showed to infect specifically *A. valerianellae* strains, producing clear plaques, thus indicating a lytic lifestyle. It was easily amplified in high concentrations and the killing curves showed to considerably reduce the bacterial population, demonstrating its ability to effectively lyse bacterial cells. Additionally, no toxins or virulence factors were found in its genome. Therefore, it was selected for further investigation in phage-based biocontrol.

Seed treatment has been proposed as a control strategy for *A. valerianellae*, since transmission by contaminated seeds is one major infection source. For instance, the use of aerated steam, hot water and sodium hypochlorite revealed to have an effect against *A. valerianellae*. However, it should be noted that due to its poor degradability and potential toxicity, sodium hypochlorite is not allowed in organic farming [80]. Alternatively, the use of bacteriophages in seed treatment could provide several advantages when compared with these available treatments, as phages are able to remain infective for long periods around the seeds, even after germination, and represent a more selective and ecologically sustainable strategy. The use of phage-coated seeds has been previously reported to be an effective approach in the biocontrol of another *Acidovorax* species, *A. citrulli*, being able to reduce the development of bacterial fruit blotch (BFB). Indeed, the treatment showed to increase the germination rate of watermelon infested seeds from 55% to 88% and the plant survival rate after three weeks from 15% to 100% [205]. As such, in this study, a seed bioassay was performed to test the efficacy of the Alfacinha3 phage in the control of *Acidovorax valerianellae* on seeds. A reduction of 87% of the bacterial concentration inside the seeds, and an increase from 58.9% to 93.3% in the germination rate were verified, showing that the phage could significantly reduce the progression of the disease. After three weeks, the non-treated infected seedlings showed an abnormal growth, in contrast to the phage-treated ones, which did not develop symptoms and were able to grow almost similarly to the non-infected plants. This demonstrates the potential of the phage Alfacinha3 in the biocontrol of *A. valerianellae*. It is worth noting that, in these experiments, the seeds were artificially infected with only one strain of *A. valerianellae*, while under natural field conditions, plants can be infected with several different strains. Thus, different combinations of phage-host should be tested, as it can possibly lead to different rates of success in reducing disease

development.

Additionally, when coating healthy seeds with the Alfacinha3 phage solution of 10^9 PFU/mL, a phage concentration of only 10^5 PFU per gram of seed was detected. This suggests a low persistence of the phage when not accompanied by a host. Even though it did not appear as an issue in suppressing the bacterial population in a treatment strategy, it can impose an obstacle in a protective/preventive framework. It is known that lower phage titers often result in lower levels of disease protection [254]. In addition, phage concentrations tend to naturally decay over time when in the plant environment, since they are more subject to environmental stresses, especially if the host is not present [255]. To improve phage persistence, the addition of non-pathogenic or attenuated phage-propagating bacterial strains has been proposed [172]. The phage stability in the seeds can also possibly be increased by incorporation into polymer-based coatings, such as polymethyl vinyl ether (PMVE), skim milk and maltodextrin [255].

Despite these results present bacteriophages as promising coating agents to treat seed-borne disease, seed contamination is not the only source of infection described for *A. valerianellae*, and so, the phage must also be able to treat the disease when developed on the foliage. Soil-based approaches have been proposed as an alternative option to foliar spraying, given the low phage persistence associated with the latter [157]. For this purpose, the translocation of the phage through the plant vascular system and the concentration that it would achieve on the leaves upon phage application in the soil should be evaluated. For instance, the absorption and transport of the *A. citrulli* phage ACPWH from soil to leaf tissue could reach a phage titer of 6.5 x 10^6 PFU/g leaf and reduce BFB disease severity by 60% in melon plants [206].

Interestingly, the plants whose seeds were phage-primed revealed significant differences in morphology. Despite shorter shoots they presented considerably longer and more developed roots, with increased lateral root density. This indicates that the interaction with the phage solution is inducing a plant response. Plants are known to respond to a variety of stimuli, including PAMPs (pathogen-associated molecular patterns), such as lipopolysaccharides, peptidoglycans and bacterial flagellin, that trigger a defense response to protect the plant from invading pathogens [256]. The detection of quorum sensing (QS) signaling molecules of microorganisms, including amino acids, fat derivatives, and other organic compounds has also been reported to induce a plant response. For instance, a recent study showed that diketopiperazines (QS signal molecules) promoted lateral root development and root hair formation in *Arabidopsis thaliana* by enhancing the polar transport of the plant hormone auxin from the shoots to the roots. This led to the accumulation of auxin at the root tip, that in turn, accelerated root growth [257]. Therefore, it is hypothesized that the phage stock used in the seed priming still contains bacterial compounds that cause a plant response. The bacterial compounds could include endotoxins, such as LPS, that could not be completely removed by PEG precipitation [239], or QS signaling molecules, that induce hormone signaling, promoting the specific growth and development of the root. As such, prior to phage application, purification methods should be applied to avoid harming plant shoots growth. For instance, alternative methods to PEG precipitation, including combinations of dead-end filtration, cross-flow filtration and affinity chromatography have been suggested [239].

In conclusion, the genomic characterization of the bacteriophages Alfacinha1, Alfacinha3 and Acica, the first isolated phages infecting *A. valerianellae* and *A. cattleyae*, revealed two novel phage genera. While the Alfacinha phages showed to display lytic lifestyle, Acica encoded toxin systems, typically correlated with lysogenic behavior, and possibly associated with lysogenic conversion. In addition, the potential of the phage Alfacinha3 in the biocontrol of *A. valerianellae* in lamb's lettuce was demonstrated, as it could significantly reduce the development and the progression of the disease.

Chapter 5

Conclusions and Future Perspectives

The growing human population, along with the limited area of cultivable land, requires an increase of current crop yields. However, plant pathogenic bacteria represent a key limiting factor. Issues concerning resistance towards the existing treatments are prominent, and therefore, there is a great need for long-term and safe alternatives. In this regard, bacteriophages are hypothesized as a potential solution to control plant diseases, and so, their applicability to combat *Acidovorax valerianellae*, the causing agent of lamb's lettuce black spot, was further investigated in this research work.

5.1 The characterization of the new *Acidovorax* isolated phages reveals a high diversity of host-phage interactions

In this study, the first phages infecting *A. valerianellae* and *A. cattleyae*, Alfacinha1, Alfacinha3, Acica and Aval were isolated and characterized. Based on their overall genome architecture, they showed to be representative of three novel genera, 'Alfacinhavirus', 'Acicavirus' and 'Avalvirus', revealing significant genomic differences to other known phages.

Within the four new phages, the host range analysis revealed diverse specificities, suggesting that they can, for instance, target different receptors or use different proteins to lyse their hosts. Both hypotheses were indeed supported by the proteome comparison, but no specific mechanism could be confirmed to explain the observed host range differences. To provide further insight about these differences, a knock-out library of the host strains using transposon mutagenesis could be used in future studies to assess which genes determine the infection mechanism in each phage. This strategy was successfully used by Holtappels *et al.* to identify host genes that affect the adsorption of the *Pseudomonas* phages KIL3b and KIL5. In addition, they were able to find a knocked-out gene, encoding an inner membrane protein, that was distinguishing the ability of KIL5 to infect the host [155]. This approach could also be very useful, when designing a cocktail, to select for phages that present different infection mechanisms, as these differences can represent an advantage by turning more difficult the selection for phage-resistant strains in the bacterial populations. Indeed, if the phages have similar infection strategies, they can compete during coinfection, reducing the access of the most effective phage, and, in turn, the efficacy of the cocktail. Consequently, a treatment based on a single phage can, in some cases, show higher disease protection compared to a phage cocktail [254]. Notably, despite the diversity observed in the host range of the *Acidovorax* phages, only 63% of the *A. valerianellae* strains collection could be infected, and the phage that was considered suitable for phage biocontrol, Alfacinha3, was able to infect only 37% of the collection. Therefore, isolation of additional phages, targeting different strains and distinct host proteins during infection, would be necessary to develop a biocontrol product relevant

for application. Another possibility could rely on the genetic modification of the temperate phage Aval to become a virulent phage, such as demonstrated by Dedrick *et al*. with a *Mycobacterium smegmatis* phage [145], and combining it with Alfacinha3, thus allowing to infect a wider array of strains.

The new isolated phages also revealed differences regarding their life cycle and genomic features. While the Alfacinha phages displayed a lytic lifestyle, Aval and Acica appeared to follow the lysogenic route. Additionally, Aval and Acica encoded toxins associated with lysogenic conversion, in contrast to both Alfacinha phages. These genes can also be passed on to other bacteria via horizontal gene transfer, and consequently select for more virulent and competitive hosts, worsening the disease transmission rather than treating it. Nevertheless, further investigation would be required to clarify the exact contribution or impact of these phages in the evolution and virulence of the *Acidovorax* strains. This might, for instance, be investigated by comparison of phenotypes between a lysogen, with an introduced temperate phage, and the parental strain lacking the prophage. In addition, although genomic characterization can predict the ability of some phages to overcome phage-targeting resistance systems, including, for instance, the expression of methyltransferases that confer protection from host restriction endonucleases, it cannot predict how the development of bacterial resistance will evolve. Therefore, a study of the long-term interaction dynamics between the phage candidate(s) and the host could provide insight into the possible development of resistance by the host and its rate. That should be taken in account when designing a phage-based product, and especially when it is based on a single phage, since bacteria are likely to develop resistance more rapidly in this case [258]. Furthermore, evaluating the emerging phage-resistant mutants could also reveal resistance mechanisms and whether they have an altered effect on virulence. Acquired phage-resistance mutations are known to be often accompanied by a secondary cost, such as attenuated pathogenicity [156, 189], and therefore this would not be necessarily detrimental in the context of phage biocontrol.

5.2 Alfacinha3 may be a promising seed-coating agent for biocontrol of *Acidovorax valerianellae*

The significant effect on seed germination and seedling development, shown in the performed bioassay, demonstrated the applicability of the Alfacinha3 phage as a seed-priming biocontrol agent. As seeds are considered one major source of infection of *A. valerianellae*, their treatment can represent a relevant strategy in reducing the emergence of this pathogen. Despite the phage has proven to prevent the progression of the disease at an early stage, it should also be able to curb it at a later stage, when transmission can occur from contaminated soil or when the pathogen enters the plant foliage. Therefore, further research should consider soil-based phage delivery and include the evaluation of the vascular uptake of phages and their translocation to the leaves. Although foliar spraying could be an alternative option, this type of treatment is often compromised by the sensitivity of phages to UV radiation [157]. Thus, following the concentration of phages over time could unravel their long-term protective effect based on different application strategies, and assess the need of protective formulations.

It was also concluded that, for phage application in seed-coating, the PEG stocks would require additional steps of purification, since they contained compounds, likely from bacterial source, that promoted the growth and development specific of the root. Therefore, further assays regarding phage purification would be important before application as a seed treatment.

Typically, in a phage-based biocontrol framework, phage stability is tested under various conditions such as temperature and pH, to assess possible limitations in infectivity in the plant environment [152]. In this research, phages were only applied in controlled conditions at a constant temperature of 16ºC. Further stability assays could be relevant when focusing field application, in which the phages are more

exposed to temperature changes and other diverse environmental conditions. Nevertheless, cultivation methods of leafy vegetables have been reformed to new cropping systems that enhance land use efficiency, such as vertical farming, in highly controlled stacking growth rooms and glasshouses. These solutions have allowed to grow 20-fold more plants per unit area than the traditional horizontal methods, increasing the crop productivity more than 10 times [259]. These systems keep a highly controlled environment, limiting, for instance, temperature fluctuations, to optimize the germination and growing rates. Specifically, for lettuce cropping, including lamb's lettuce, temperatures do not significantly deviate from 16°C, for which the application of the phage Alfacinha3 proved to perform well in this research work.

Furthermore, phage-based products could be implemented along with smart farming techniques, that integrate robotics, machine learning and sensor-based technologies to exploit early disease detection and precise biocontrol. Hyperspectral sensors trained with machine learning algorithms can be used for the detection of different phytopathogens [260, 261], which could then be specifically targeted by bacteriophages, possibly combined with other biocontrol organisms or biopesticides, in an automated and precise manner. In this perspective, phage application could be customized to meet the specific needs of both phages and crops, allowing to fully exploit the benefits of an integrated biocontrol strategy.

5.3 General conclusion

In conclusion, this research project contributed to expand knowledge of new bacteriophages infecting *Acidovorax* phytopathogens, and to explore their potential as a biocontrol tool.

Genomic analyses allowed to obtain a thorough understanding about phage biology and its interactions with the host, representing a fundamental tool to ensure safe phage application. Combining this extensive knowledge along with the examination of the practical applicability of phages in different settings, would help to improve and optimize a phage-based biocontrol strategy.

In the future, much more attention should be provided to sustainable pest control, in order to boost crop yield with a more rational application of resources. Global crop production must continue to grow to keep up with the predicted population growth and consumption trends. However, that should not impose an agricultural expansion of croplands. Integrated pest management techniques, combining the use of biological control agents, such as bacteriophages, with optimized cultural practices, including vertical and smart farming, can be used to achieve an increased potential of the available agricultural areas. In addition, phages tend to persist in high numbers only as long as the host is present, without presenting accumulation in the soil like copper-based or other chemical pesticides. Therefore, bacteriophages, as naturally occurring bacterial predators, can play a revolutionary role in a more sustainable future for crop production.

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Appendix A

Plaque Morphologies

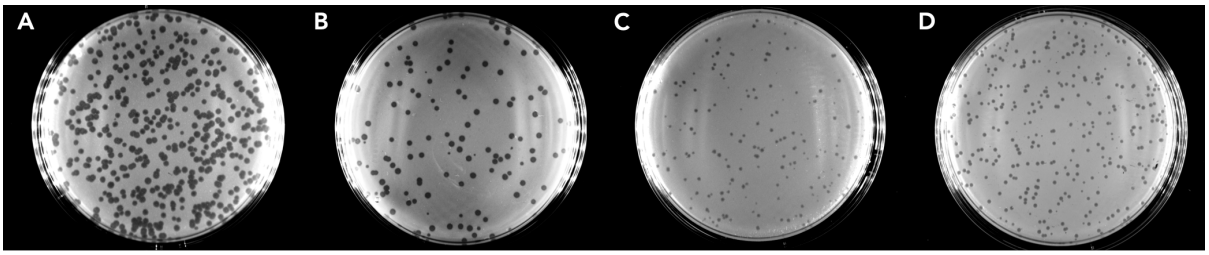

Figure A.1: Plaque morphologies of the four phages Alfacinha1 (A), Alfacinha3 (B), Aval (C) and Acica (D). Alfacinha1 and Alfacinha 3 show small plaques (2-3 mm) and Aval and Acica very small plaques ($<$2 mm).

Appendix B

Phage Genome Annotations

Table B.1: Annotation of the genome of the bacteriophage Aval.

ORF	Start	Stop	Strand	Top BLAST hit
1	82	1542	+	Integrase arm-type DNA-binding domain-containing protein
2	1771	1514	-	AlpA family phage regulatory protein
3	2004	1768	-	hypothetical protein
4	3146	2004	-	hypothetical protein
5	3592	3143	-	hypothetical protein
6	6371	3510	-	Chromosome (plasmid) partitioning protein ParB
7	6673	6374	-	hypothetical protein
8	7002	6691	-	hypothetical protein
9	7194	7021	-	hypothetical protein
10	7322	7191	-	hypothetical protein
11	7699	7319	-	hypothetical protein
12	7899	7699	-	hypothetical protein
13	8699	8037	-	Helix-turn-helix domain-containing protein
14	9180	9656	+	Phage activator protein cII
15	9794	9946	+	hypothetical protein
16	10028	12706	+	Virulence protein E
17	12693	12845	+	hypothetical protein
18	13071	13427	+	hypothetical protein
19	13414	13836	+	hypothetical protein
20	14549	13932	-	hypothetical protein
21	14588	14971	+	HNH endonuclease
22	15154	15723	+	P27 family phage terminase small subunit
23	15730	17466	+	Terminase large subunit
24	17463	18719	+	Phage portal protein
25	18719	19354	+	HK97 family phage prohead protease
26	19357	20616	+	Phage major capsid protein
27	20688	21125	+	hypothetical protein
28	21350	22354	+	HK97 gp10 family phage protein: putative tail-component
29	22351	22707	+	hypothetical protein
30	22788	23441	+	Phage tail protein
31	23541	23960	+	Phage tail assembly chaperone
32	23978	24289	+	FAD-dependent oxidoreductase
33	24338	24793	+	hypothetical protein

ORF	Start	Stop	Strand	Top BLAST hit
34	24871	30423	+	Phage tail length tape measure family protein H
35	30456	31364	+	hypothetical protein
36	31361	32449	+	Pyocin knob domain-containing protein
37	32413	33840	+	hypothetical protein
38	33842	34465	+	hypothetical protein
39	34530	34946	+	Structural protein P5
40	34946	35332	+	Methyl-accepting chemotaxis protein
41	35319	35729	+	hypothetical protein
42	35726	35938	+	hypothetical protein
43	35935	36300	+	Lipopolysaccharide assembly protein A domain
44	36239	37501	+	DNA adenine methylase
45	37750	38259	+	hypothetical protein
46	38688	38497	-	hypothetical protein
47	39146	38685	-	hypothetical protein
48	39439	39143	-	hypothetical protein

Table B.2: Annotation of the genome of the bacteriophage Alfacinha1.

ORF	Start	Stop	Strand	Top BLAST hit
1	1254	160	-	Phage portal vertex protein GpQ
2	3305	1251	-	Phage terminase, ATPase subunit GpP
3	3247	4134	+	Phage capsid scaffolding protein GpO
4	4196	5200	+	Phage major capsid protein, P2 family
5	5295	6020	+	Phage terminase, endonuclease subunit GpM
6	6126	6608	+	Phage head completion/stabilization protein
7	6605	6823	+	Phage tail protein X
8	6827	7033	+	Phage holin T7 family
9	7161	7724	+	Lysozyme
10	7842	8384	+	hypothetical protein
11	8386	8901	+	Phage tail protein
12	8905	9378	+	Phage virion morphogenesis protein
13	9765	11267	+	Baseplate J/gp47 family protein
14	11267	12187	+	Phage tail protein
15	12381	15941	+	putative VrlC protein
16	15951	17435	+	Phage tail protein
17	17432	17818	+	hypothetical protein
18	17837	18577	+	Envelope protein
19	18677	19009	+	hypothetical protein
20	19006	19251	+	hypothetical protein
21	19214	19549	+	hypothetical protein
22	19546	19755	+	Helix-turn-helix transcriptional regulator
23	19868	21112	+	Phage tail sheath subtilisin-like domain-containing protein
24	21158	21667	+	Phage major tail tube protein
25	21790	22125	+	Phage tail assembly protein
26	22176	22265	+	GpE family phage tail protein

ORF	Start	Stop	Strand	Top BLAST hit
27	22318	24969	+	Phage tail length tape-measure protein GpT
28	24997	25458	+	Phage tail protein
29	25462	26517	+	Phage late control D family protein
30	27002	26562	-	hypothetical protein
31	28163	27390	-	Helix-turn-helix transcriptional regulator
32	28209	28430	+	hypothetical protein
33	28450	28731	+	hypothetical protein
34	28751	28912	+	hypothetical protein
35	28958	29404	+	ogr/Delta-like zinc finger family protein
36	29521	29946	+	ABC transporter ATP-binding protein
37	29937	30089	+	hypothetical protein
38	30086	30346	+	hypothetical protein
39	30349	30507	+	hypothetical protein
40	30504	30911	+	hypothetical protein
41	30926	32884	+	Phage replication protein GpA, endonuclease
42	32884	33156	+	hypothetical protein
43	33172	33390	+	hypothetical protein
44	33418	33681	+	hypothetical protein
45	33685	33915	+	hypothetical protein
46	33915	35048	+	Cytosine-specific methyltransferase
47	35045	35956	+	hypothetical protein
48	35953	36210	+	ABC transporter substrate-binding protein
49	36427	37806	+	Phosphoadenosine phosphosulfate reductase family protein
50	37815	38345	+	hypothetical protein
51	38345	38545	+	AlpA family phage regulatory protein
52	38609	38848	+	hypothetical protein
53	39877	38843	-	Site-specific integrase

Table B.3: Annotation of the genome of the bacteriophage Alfacinha3.

ORF	Start	Stop	Strand	Top BLAST hit
1	1318	224	-	Phage portal vertex protein GpQ
2	3226	1322	-	Phage terminase, ATPase subunit GpP
3	3312	4199	+	Phage capsid scaffolding protein GpO
4	4262	5266	+	Phage major capsid protein, P2 family
5	5360	6085	+	Phage terminase, endonuclease subunit GpM
6	6191	6673	+	Phage head completion/stabilization protein
7	6670	6888	+	Phage tail protein X
8	6892	7098	+	Phage holin T7 family
9	7098	7667	+	Lysozyme
10	7785	8327	+	hypothetical protein
11	8329	8844	+	Phage tail protein
12	8848	9321	+	Phage virion morphogenesis protein
13	9708	11210	+	Baseplate J/gp47 family protein
14	11210	12130	+	Phage tail protein

ORF	Start	Stop	Strand	Top BLAST hit
15	12324	15884	+	Putative VrlC protein
16	15894	17378	+	Phage tail protein
17	17375	17761	+	hypothetical protein
18	17780	18520	+	Envelope protein
19	18620	18952	+	hypothetical protein
20	18949	19194	+	hypothetical protein
21	19157	19492	+	hypothetical protein
22	19489	19698	+	Helix-turn-helix transcriptional regulator
23	19811	21055	+	Phage tail sheath subtilisin-like domain-containing protein
24	21101	21610	+	Phage major tail tube protein GpFII
25	21733	22068	+	Phage tail assembly protein
26	22119	22208	+	GpE family phage tail protein
27	22261	24912	+	Phage tail length tape-measure protein GpT
28	24940	25401	+	Phage tail protein
29	25405	26460	+	Phage late control D family protein
30	26945	26505	-	hypothetical protein
31	28157	27333	-	Helix-turn-helix transcriptional regulator
32	28151	28372	+	hypothetical protein
33	28392	28673	+	hypothetical protein
34	28693	28854	+	hypothetical protein
35	28924	29346	+	ogr/Delta-like zinc finger family protein
36	29463	29888	+	ABC transporter ATP-binding protein
37	29879	30031	+	hypothetical protein
38	30028	30288	+	hypothetical protein
39	30291	30449	+	hypothetical protein
40	30446	30853	+	hypothetical protein
41	30868	32826	+	Phage replication protein GpA, endonuclease
42	32826	33098	+	hypothetical protein
43	33114	33332	+	hypothetical protein
44	33360	33623	+	hypothetical protein
45	33627	33857	+	hypothetical protein
46	33857	35023	+	Cytosine-specific methyltransferase
47	35020	36024	+	hypothetical protein
48	36021	36278	+	ABC transporter substrate-binding protein
49	36495	37874	+	Phosphoadenosine phosphosulfate reductase family protein
50	37883	38413	+	hypothetical protein
51	38413	38613	+	AlpA family phage regulatory protein
52	38677	38916	+	hypothetical protein
53	40104	38911	-	Site-specific integrase

Table B.4: Annotation of the genome of the bacteriophage Acica.

ORF	Start	Stop	Strand	Top BLAST hit
1	1096	23	-	Phage portal vertex protein GpQ
2	2940	1102	-	Phage terminase, ATPase subunit GpP
3	2779	4962	+	Phage major capsid protein, P2 family
4	5050	5754	+	Phage terminase, endonuclease subunit GpM
5	5868	6350	+	Phage head completion-stabilization protein
6	6347	6568	+	Phage tail protein X
7	6605	6937	+	Type II Holin
8	6937	7569	+	Endolysin
9	7569	8156	+	hypothetical protein
10	8122	8691	+	Phage tail protein
11	8695	9165	+	Phage virion morphogenesis protein
12	9240	9836	+	Phage baseplate assembly protein V
13	9836	10198	+	Phage baseplate assembly chaperone
14	10195	11046	+	Baseplate J/gp47 family protein
15	11043	11975	+	Phage tail protein
16	12124	15738	+	putative VrlC protein
17	15707	16234	+	RHS repeat protein
18	16231	17091	+	hypothetical protein
19	17097	17411	+	hypothetical protein
20	17561	18757	+	Phage tail sheath subtilisin-like domain-containing protein
21	18793	19302	+	Phage major tail tube protein
22	19399	19746	+	Phage tail assembly protein
23	19755	19886	+	GpE family phage tail protein
24	19900	22545	+	Phage tail protein
25	22570	23034	+	Phage tail protein
26	23037	24095	+	Phage late control D family protein
27	24149	24418	+	BrnT family toxin
28	24402	24698	+	BrnA antitoxin family protein
29	25227	24709	-	hypothetical protein
30	25913	25245	-	helix-turn-helix transcriptional regulator
31	26026	26253	+	hypothetical protein
32	26271	26498	+	hypothetical protein
33	26491	26760	+	hypothetical protein
34	26828	27250	+	ogr/Delta-like zinc finger family protein
35	27368	27790	+	hypothetical protein
36	27930	28091	+	hypothetical protein
37	28133	28411	+	hypothetical protein
38	28415	30358	+	Phage replication protein GpA, endonuclease
39	30358	30642	+	hypothetical protein
40	30639	30860	+	hypothetical protein
41	30896	31171	+	hypothetical protein
42	31175	31402	+	hypothetical protein
43	31422	32132	+	hypothetical protein
44	32129	32779	+	hypothetical protein
45	32776	33699	+	C-5 cytosine-specific DNA methylase family protein
46	33735	34835	+	Phosphoadenosine phosphosulfate reductase family protein
47	34832	35068	+	AlpA family phage regulatory protein
48	36306	35035	-	Phage integrase, site-specific tyrosine recombinase
49	37472	37008	-	hypothetical protein