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Posted Date: 30 July 2024

doi: 10.20944/preprints202407.2228.v1

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Isolation, Characterization and Genomic Analysis of a Novel Lytic Bacteriophage PAT1 Infecting *Agrobacterium tumefaciens*

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Abstract: *Agrobacterium tumefaciens* (*A. tumefaciens*) is a plant pathogenic bacterium that causes crown gall disease, leading to significant economic losses in various agriculturally important crops, including apple, pear, peach, and almond. The difficulty of controlling *A. tumefaciens* with conventional pesticides underscores the need for alternative antibacterial agents such as bacteriophages. In this study, a novel lytic bacteriophage, named *Agrobacterium* phage PAT1 (PAT1), with high lysis potential against *A. tumefaciens*, was isolated from wastewater. The interaction between PAT1 and *A. tumefaciens* cells, investigated using Transmission electron microscopy, revealed that PAT1 efficiently adsorb, infect, and replicate on *A. tumefaciens* in a very short time period (i.e., ≤30 min for a complete infectious cycle). Furthermore, the turbidity assay showed that PAT1 (MOI = 1) exerted a significant inhibitory effect on *A. tumefaciens* for 48 hours, resulting in an 82% reduction in bacterial growth. Additionally, stability tests showed that PAT1 was highly resistant to a broad range of pH (4-10) and temperatures (4-60 °C). Bioinformatics analyses of the genomic sequence suggested that PAT1 is a new phage closely related to *Agrobacterium* phages of the genus *Atuphduovirus* within the family *Autographiviridae*. The genome size was 45,040 base pairs with a G+C content of 54.5%, consisting of 54 coding sequences (CDS), of which the functions of 22 CDS were predicted, including two endolysins genes which could be used as antimicrobials against *A. tumefaciens*. Additionally, no lysogenic mediated genes or genes encoding virulence factors, antibiotic resistance, or toxins were detected in PAT1 genome. Overall, PAT1 demonstrated suitability as a potential biocontrol agent for combating *A. tumefaciens* infections, expanding the limited catalog of such lytic *A. tumefaciens* phages. However, its efficacy in controlling crown gall disease *in planta* remains to be evaluated.

Keywords: plant pathogenic bacteria; lytic bacteriophage; biocontrol; characterization; genomic analysis

1. Introduction

Agrobacterium tumefaciens (*A. tumefaciens*) is a Gram-negative, non-spore-forming, motile, rod-shaped plant pathogenic bacterium that causes crown gall disease in a wide range of plant species worldwide [1]. Crown gall compromises the commercialization of plants in more than 60 families, including dicotyledonous plants, ornamental plants, brambles, and woody plants such as pome fruit, stone fruit, and nut trees [1–3]. *A. tumefaciens* is commonly found in the rhizosphere of many plants, where it survives on root exudates [4]. It enters the plant through wounds on the roots, stems and crowns, which often occur in orchards during pruning and in nurseries through transplanting and grafting [1,4]. Upon entering the plant, this pathogen pursues a pathogenic lifestyle by transforming plants with a fragment of the tumor-inducing (Ti) plasmid, the transfer-DNA (T-DNA), which induces the abnormal proliferation of plant cells via the synthesis of phytohormones, leading to the

 doi:10.20944/preprints202407.2228.v1

formation of tumors (or galls) [5,6]. Galls usually develop at the crown but can also occur on secondary or lateral roots as well as the main stem and branches above the soil line, restricting the flow of water and nutrients within the plant. This obstruction leads to significant yield losses and, in severe cases, plant death [4,7].

To date, there are no synthetic chemical treatments for controlling crown gall. Eco-friendly management of this disease using biocontrol agents such as the non-pathogenic *Agrobacterium radiobacter* strain K84 and its genetically modified strain K1026 has proven to be an effective method in several locations [8]. However, K84 and K1026 are ineffective against certain strains of *A. tumefaciens*; thereby limiting their ability to provide broad-spectrum control [9]. Therefore, there is a critical need to identify new effective biocontrol agents against *A. tumefaciens*. In this regard, virulent (lytic) bacteriophages, which are viruses that specifically infect and lyse bacteria, are certainly among the most attractive options in the field of biological control. Lytic bacteriophages are ubiquitous in the environment, recognized as safe agents, and their role as potent antibacterial agents in agriculture has been well documented [10–12]. Lytic phages exhibit many advantageous characteristics as biocontrol agents, including ease of discovery, high host-bacteria specificity, self-replicating nature, harmlessness to eukaryotes, low environmental impact, low cost and simple process for preparation, high efficiency at low multiplicity of infection (MOI), and their post-application levels will increase at the expense of bacterial host survival, instead of decreasing as is the case for other types of antimicrobial compounds [13,14].

In the case of *A. tumefaciens*, there are only seven characterized lytic phages reported in the literature to infect this bacterial plant pathogen: 7-7-1 [15], Atu_ph02 and Atu_ph03 [16], Atu_ph07—a jumbo phage [17], Atu_ph04 and Atu_ph08 [18], and Milano [19]. In the context of expanding the number of phages infecting *A. tumefaciens*, this study reports the isolation and characterization of a novel lytic phage of *A. tumefaciens*, named *Agrobacterium* phage PAT1 (PAT1), which demonstrated significant antibacterial efficacy against *A. tumefaciens* in-vitro.

2. Materials and Methods

2.1. Bacterial Strains and Culture Conditions

Bacteria listed in Table 1 were grown either at 28 °C in liquid yeast extract peptone glucose broth (YPG) (5.0 g/L yeast extract, 5.0 g/L peptone and 10.0 g/L glucose) or on yeast extract peptone glucose agar (YPGA, i.e., YPG supplemented with 1.5% agar).

Table 1. Bacterial strains used for PAT1 host range determination.

Species	Isolate	Host plant	Origin
Xanthomonas campestris pv. *campestris*	CFBP 1710	*Brassica oleracea var. botrytis*	France
Xanthomonas albilineans	CFBP 1943	-	Burkina Faso
Erwinia amylovora	PGL Z1*	*Pyrus communis*	Italy
Pseudomonas syringae pv. *syringae*	CFBP 311	*Pyrus communis*	France
Dickeya chrysanthemi biovar *chrysanthemi*	CFBP 1346	*Chrysanthemum maximum*	Italy
Pseudomonas savastanoi pv. *savastanoi*	CFBP 5050	*Olea europaea*	Portugal
Agrobacterium larrymoorei	CFBP 5473	*Ficus benjamina*	USA
Agrobacterium rubi	CFBP 5521	*Rubus sp.*	Germany
Agrobacterium tumefaciens	CFBP 5770	*Prunus persica*	Australia
Agrobacterium tumefaciens	YD 5156-2018	*Prunus domestica*	Greece
Agrobacterium tumefaciens	YD 5660-2007	*Prunus dulcis*	Greece
Agrobacterium tumefaciens	BPIC 139	*Vitis vinifera*	Greece
Agrobacterium tumefaciens	BPIC 284	*Prunus dulcis*	Greece
Agrobacterium tumefaciens	BPIC 310	*Pyrus amygdaliformis*	Greece
Agrobacterium vitis	CFBP 2738	*Vitis vinifera*	Greece
Agrobacterium vitis	BPIC 1009	*Vitis vinifera*	Greece

* Collection of CIHEAM-IAM, Bari, Italy. CFBP: French Collection of Phytopathogenic Bacteria, Angers, France. YD: Collection of bacterial strains isolated in the frame of the diagnostic work of the

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laboratory of bacteriology of Benaki phytopathological institute. BPIC: Benaki phytopathological institute collections.

2.2. Bacteriophage Isolation, Purification, and Titration

The phage described in this study was isolated from a sewage water sample collected at the untreated influx point of the wastewater processing station in Bari (south of Italy), in April 2023. Briefly, 1 L of sewage water was passed through a filter paper of Grade 1, Dia. 75 × 100 mm (Whatman, Maidstone, UK) to remove large particles and filtered through a 0.22 µm filter (Merck, Rome, Italy) to remove cellular debris. The filtrate was centrifuged at 108,763 g (Rotor J50.2 Ti, Beckmann Coulter, Ca, USA) for 1h at 4 °C to pellet phage particles. Pellets were resuspended in 2 mL phage buffer (100 mM Tris-HCl (pH 7.6); 10 mM $MgCl_2$; 100 mM NaCl; and 10 mM $MgSO_4$) and stored at 4 °C. For phage enrichment, *A. tumefaciens* strain CFBP 5770 was grown at 28 °C on YPG agar medium for 24h and transferred to 2 mL YPG broth medium at an optical density at 600 nm (OD_{600}) of 0.1, to which 100 µL of pre-treated sample were added. The culture enrichment was incubated at 28 °C for 24h. Phage was purified from filtrate using the standard double agar overlay method [20]. Single clear plaque-forming unit was transferred into 1 mL of phage buffer and this process was repeated three times to ensure the isolation of a single phage. To obtain high phage titer, 1 mL of *A. tumefaciens* strain CFBP 5770 culture at an optical density at 600 nm (OD600) of 0.2 was inoculated in 500 ml YPG broth medium and 1 mL of purified phage was added, and the mix was incubated for up to 24 h at 28 °C. Amplified phages were filtered through 0.22 µm filters, concentrated by high-speed centrifugation (108,763 g for 1h), resuspended in 2 mL of phage buffer, and stored at 4 °C for further analysis. The phage titer was determined through a double-layer assay.

2.3. Spot Assay

The lytic activity of PAT1 against *A. tumefaciens* was assessed using a spot assay as follows: 200 µL of *A. tumefaciens* strain CFBP 5770 suspension (10^8 CFU/mL) were mixed with 6 mL of YPG soft agar (i.e., YPG supplemented with 0.7% agar), poured into YPGA plates, and allowed to dry. Subsequently, drops of 10 µL of phage solution at 10^8, 10^7, 10^6, 10^5, and 10^4 PFU/mL were spotted onto the surface of the plates. Spots were dried at room temperature and the plates cultured for 24 h at 28 °C.

2.4. Transmission Electron Microscopy (TEM)

To scrutinize the morphological and lytic properties of the purified phage PAT1, a culture of *A. tumefaciens* strain CFBP 5770 was challenged with PAT1 (multiplicity of infection, MOI = 1) for 1h at room temperature. Representative images of the phage and bacterial cells were taken at 10-, 30-, and 60-min post-infection (pi) via TEM (FEI MORGAGNI 282D, USA) using the dip method. Briefly, carbon-coated copper/rhodium grids underwent a 2-minute incubation period with either the phage alone or the phage-treated cells, followed by rinsing with 200 µL of distilled water. Negative staining was obtained by immersing the grids in 200 µL of a 0.5 % w/v UA-Zero EM stain solution (Agar-Scientific Ltd., Stansted, UK), and observed under an accelerating voltage of 80 kV.

2.5. DNA Extraction, Whole-Genome Sequencing, and Bioinformatic Analysis

Genomic DNA of PAT1 was extracted from a high-titer stock of phage particles at ~10^{10} PFU/mL using a DNeasy Plant Extraction kit following the manufacturer's protocol (Qiagen, Milan, Italy). The extracted DNA was quantified using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA). Subsequently, 500 ng of purified genomic DNA was sent for Illumina sequencing (2 × 150 bp paired-end mode) (Eurofins Genomics, Germany). The reads were quality checked and trimmed and *de novo* assembled using the Tadpole tool with different k-mers (Geneious Prime 2024.0.3, San Diego, CA, USA). The ORFs functions were annotated with Geneious, and the predictions of antibiotic resistance genes, acquired virulence genes, and toxin-encoding genes were assessed by CGE (http://www.genomicepidemiology.org/). The

 doi:10.20944/preprints202407.2228.v1

complete genome sequence of PAT1 was deposited at GenBank and a circular map of the genome and phylogenetic tree were constructed by utilizing ViPTree [21].

2.6. Optimal Multiplicity of Infection (MOI) of Phage

To investigate the phage's ability to inhibit the growth of *A. tumefaciens* in liquid growth medium, phage PAT1 and host bacterial strain CFBP 5770 were mixed at MOIs of 1, 0.1, 0.01, 0.001, and 0.0001. The mixture was then inoculated in 2 mL of YPG broth and incubated at 28 °C for 48 h. During incubation, four optical density (OD) measures (0 min, 6 h, 24 h, and 48 h) at OD600 were taken using the NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer.

2.7. Host Range Analysis

Host range of PAT1 was determined using the phage sensitivity spot test as described earlier. Briefly, bacterial strains listed in Table 1 were cultured at 28 °C on YPGA plates for up to 2 days. Then, the cultures were suspended in sterile distilled water and 200 µL of bacterial suspension (OD_{600} = 0.2) were mixed with 6 mL of YPG soft agar, poured into YPGA plates, and allowed to dry. Subsequently, drops of 10 µL of phage solution at 10^8, 10^7, 10^6, 10^5, and 10^4 PFU/mL were spotted onto the surface of the plates. Spots were dried at room temperature and the plates cultured for up to 2 days at 28 °C. The presence of a clear zone was recorded as the strain being susceptible to PAT1.

2.8. Temperature and pH Stability

The thermal stability of PAT1 was assessed by incubating 100 µL of phage suspension (~10^7 PFU/mL) at 4, 28, 40, 50, 60, and 70 °C for 1h. Following the incubation period, a serial dilution was made with phage buffer, and phage titers were determined using double agar overlay method. To assess pH stability, 100 µL of the phage suspensions was added to 900 µL of sterile-filtered YPG medium that was pH adjusted using 1 M NaOH or 1 M HCl and incubated at 28 °C for 1 h. Subsequently, a serial dilution was made with phage buffer, and phage titers were determined using double agar overlay method.

3. Results

3.1. Spot Assay

The antibacterial activity of the newly discovered phage (PAT1) against *A. tumefaciens* was initially examined through a spot assay. The results showed that PAT1 produced clear lysis zones on the *A. tumefaciens* lawn at all tested titers, highlighting the lysis potential of PAT1 against *A. tumefaciens* (Figure 1).

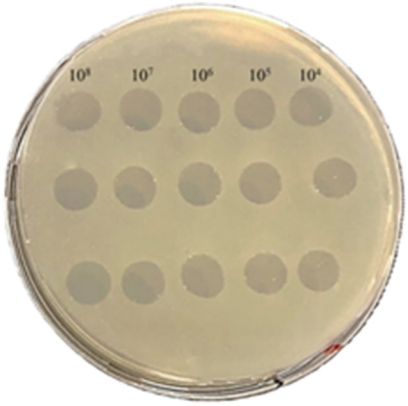

Figure 1. YPGA agar plate showing the antibacterial activity of PAT1 at different titers (10^8-10^4 PFU/mL) against *A. tumefaciens* in triplicates.

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3.2. Morphological and Lytic Properties of PAT1

PAT1 produced large clear plaques, ranging in size from 2 to 5 mm in diameter, on a lawn culture of the *A. tumefaciens* host strain CFBP 5770 (Figure 2A). TEM analysis showed that PAT1 has the typical morphological features of a podovirus morphotype C1 with an icosahedral and Head-tail geometries, the capsid diameter is around 60 ± 3 nm (length/width ratio=1) and the non-contractile tail is 10 ± 2 nm in length (Figure 2B). TEM was also used to explore at the ultrastructural level the virulence of PAT1 against *A. tumefaciens*. Micrographs showed the adsorption of PAT1 on the cell surface of *A. tumefaciens* in 10 min pi (Figure 3B), while the lysed cells of *A. tumefaciens* and the release of progeny virions from infected bacteria were visualized 30 min pi (Figure 3C,D). These observations demonstrated the ability of PAT1 to efficiently adsorb, replicate and kill *A. tumefaciens* in a very short time (*i.e.*, <30 min for a complete infectious cycle), indicating a lytic infection cycle.

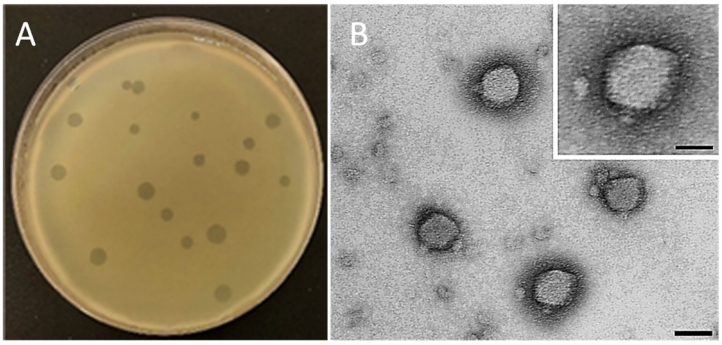

Figure 2. (**A**) Plaque morphology of PAT1 formed on *A. tumefaciens* double layer agar plate. (**B**) Transmission electron microscopy image of PAT1 showing a particle with an icosahedral capsid and a very short non-contractile tail. Scale bar: 50 nm, inset 25 nm.

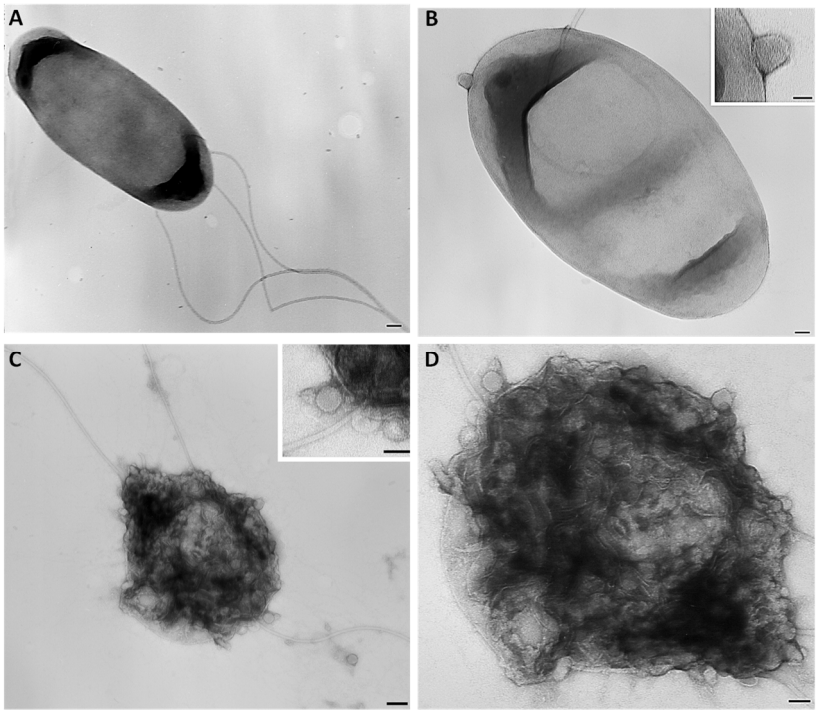

Figure 3. Transmission electron micrographs of *A. tumefaciens* cells challenged with PAT1. (**A**) untreated *A. tumefaciens* cells, used as control. (**B**) PAT1 attachment on *A. tumefaciens* cell surface, inset shows the point of phage penetration. (**C** and **D**) Lysis of PAT1-treated *A. tumefaciens* cells with release of phage progeny (inset in C). Scale bar: A, C 100 nm; B, D 50 nm; inset B 25 nm; C 50 nm.

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3.3. Phage Stability and Host Range Determination

The thermal and pH stabilities of PAT1 were estimated by measuring variations in survival rates as a function of the number of plaque-forming units (PFU). The phage was generally stable at temperatures ranging from 4 °C to 60 °C, whereas it had approximately tenfold lower infectivity at 60 °C and incubation at 70 °C for 1h killed it (Figure 4A). Furthermore, PAT1 demonstrated stable infectivity in the pH range of 4 to 10 (Figure 4B).

Host range analysis of PAT1 performed on 16 different bacterial strains (Table 1) revealed that the two strains of *A. tumefaciens* (CFBP 5770 and BPIC 284) were susceptible equally to PAT1, producing clear lysis zones on them at different tested titers (data not shown). However, PAT1 was inactive against other *A. tumefaciens* strains, and the plant pathogenic bacteria examined, indicating that this phage is likely specific to certain *A. tumefaciens* strains.

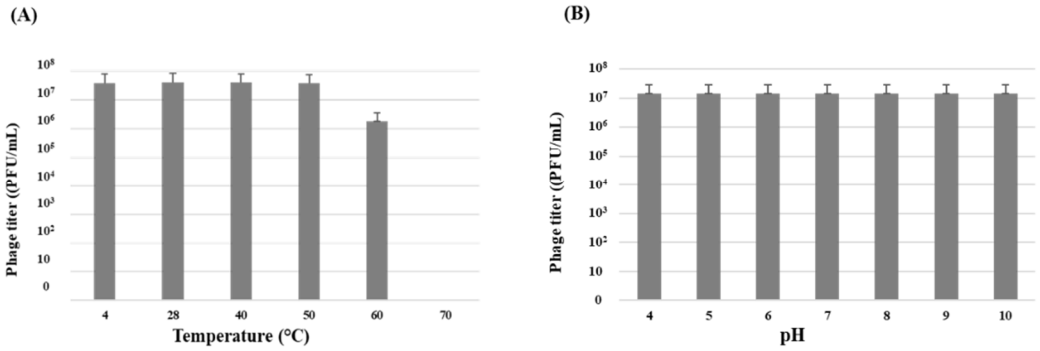

Figure 4. Histograms showing thermal and pH stability tests of PAT1. (**A**) Phage infectivity after being treated with different temperatures for 60 min. (**B**) Phage infectivity after incubation at different pHs for 60 min. Phage titers were determined using double agar overlay method. Error bars represent standard deviations from three replicates.

3.4. Bacteriolytic Effect of PAT1 on A. tumefaciens Growth

The ability of PAT1 to restrict the growth of *A. tumefaciens* strain CFBP 5770 was determined at different MOIs (1, 0.1, 0.01, 0.001, and 0.0001). All MOIs were efficient, and the phage was able to effectively restrict the growth of *A. tumefaciens* for 24h (Figure 5). However, after 24h, PAT1-infected bacteria started to display an increase in OD reading, indicating the emergence of phage-resistant mutants. Additionally, bacteria treated with the higher MOI (1) showed a slower increase in OD readings after 24h (Figure 5); therefore, it was determined to be the optimal MOI of PAT1. Despite the resistance development, PAT1 (MOI = 1) showed considerable antibacterial activity against *A. tumefaciens* growth, achieving an 82% reduction in bacterial growth, 48h pi. At the end of the experiments, PAT1-resistant mutants were isolated on YPGA agar plates and resistance was confirmed by spot assay. These results shows that PAT1 possesses an effective inhibitory potency against *A. tumefaciens*, indicating its potential implication for controlling crown gall.

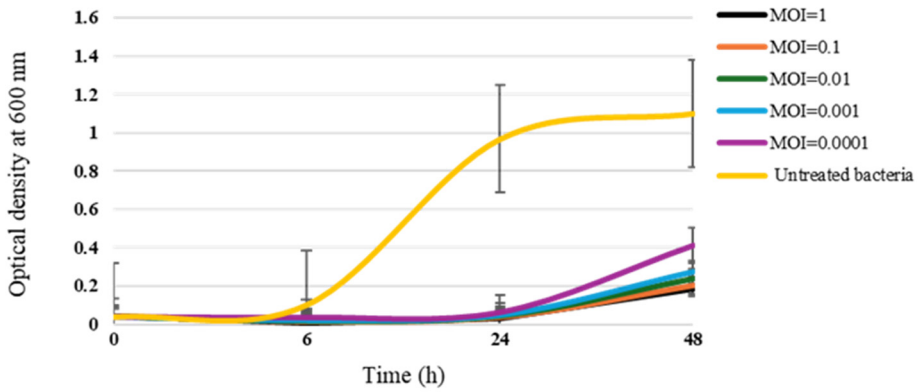

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Figure 5. Growth curve of *A. tumefaciens* treated with PAT1 at different MOIs. The ODs of treated and untreated-bacterial culture with PAT1, 48 h pi, are compared. The bars show the averages for three replicates.

3.5. Genomic and Phylogenetic Analyses of PAT1

The whole genome sequencing and *de novo* assembly of PAT1 revealed a double-stranded DNA genome of 45,040 base pairs in length with a G + C content of 54.5%, which is lower than that of *A. tumefaciens* (average 58.5%) [22]. The complete genome of PAT1 consisted of 54 coding sequences (CDSs), of which 32 (59.3%) encode for hypothetical proteins, while the function of 22 CDSs (40.7%) could be predicted. The latter encode proteins involved in DNA replication and regulation; DNA packaging and structural proteins; and cell lysis as highlighted on the genomic map (Figure 6). CGE analysis showed that the PAT1 genome does not harbor any known genes associated with antibiotic resistance, lysogenic, toxins or other virulence factors, indicating its suitability for use as a biocontrol agent.

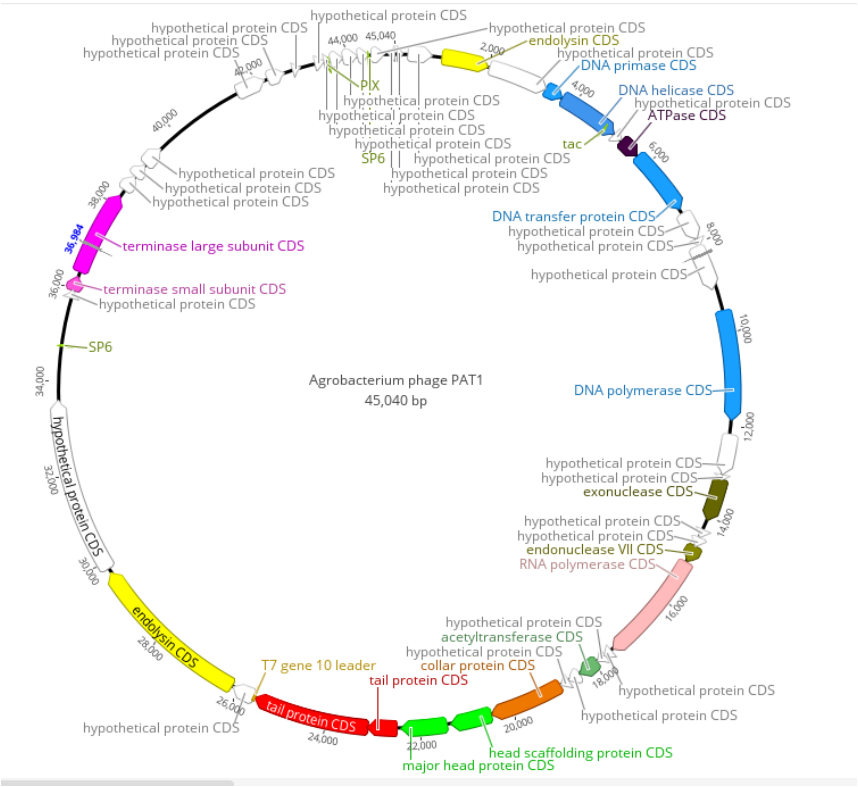

Figure 6. Circular genomic map of PAT1 representing 54 coding sequences encoded by the genome. Hypothetical proteins are displayed in grey and the predicted proteins with a signed functions are highlighted on the genomic map. The map was generated using Geneious Prime 2024.0.3.

In addition, genome sequence analysis showed that PAT1 shares maximum nucleotide identity with *Agrobacterium* phage Atu_ph02 (78.7%; accession n°: NC_047845) and *Agrobacterium* phage Atu_ph03 (78.5%; accession n°: NC_047846) (Figure 7), which are both members of the genus *Atuphduovirus* in the family *Autographiviridae* and known to infect *A. tumefaciens*. Based on the demarcation criteria of the *"International Committee on Taxonomy of Viruses"* (ICTV) for the classification of new bacteriophages species (sequence identity ≤ 95%), PAT1 is a putative new species and accordingly named as *Agrobacterium* phage PAT1.

The compete genome sequence of PAT1 was deposited in the GenBank under the accession n°: 2853494. Furthermore, the proteomic tree of PAT1 genome sequence along with its close homologues and outliers based on genome-wide sequence similarities computed by tBLASTx allocated PAT1 in a clade with *Agrobacterium* phage Atu_ph02 and *Agrobacterium* phage Atu_ph03 classified in the genus

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Atuphduovirus within the family *Autographiviridae* (Figure 8); therefore, PAT1 is to be considered as a tentative novel member of the genus *Atuphduovirus*.

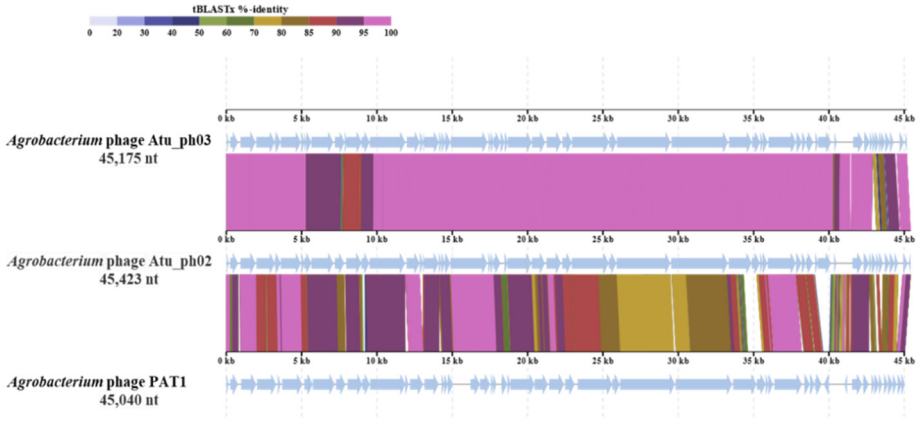

Figure 7. Genomic alignment of PAT1 with its close homologues. The colored vertical blocks between the genomes indicate the level of nucleotide similarity. The genome alignment was generated using ViPTree.

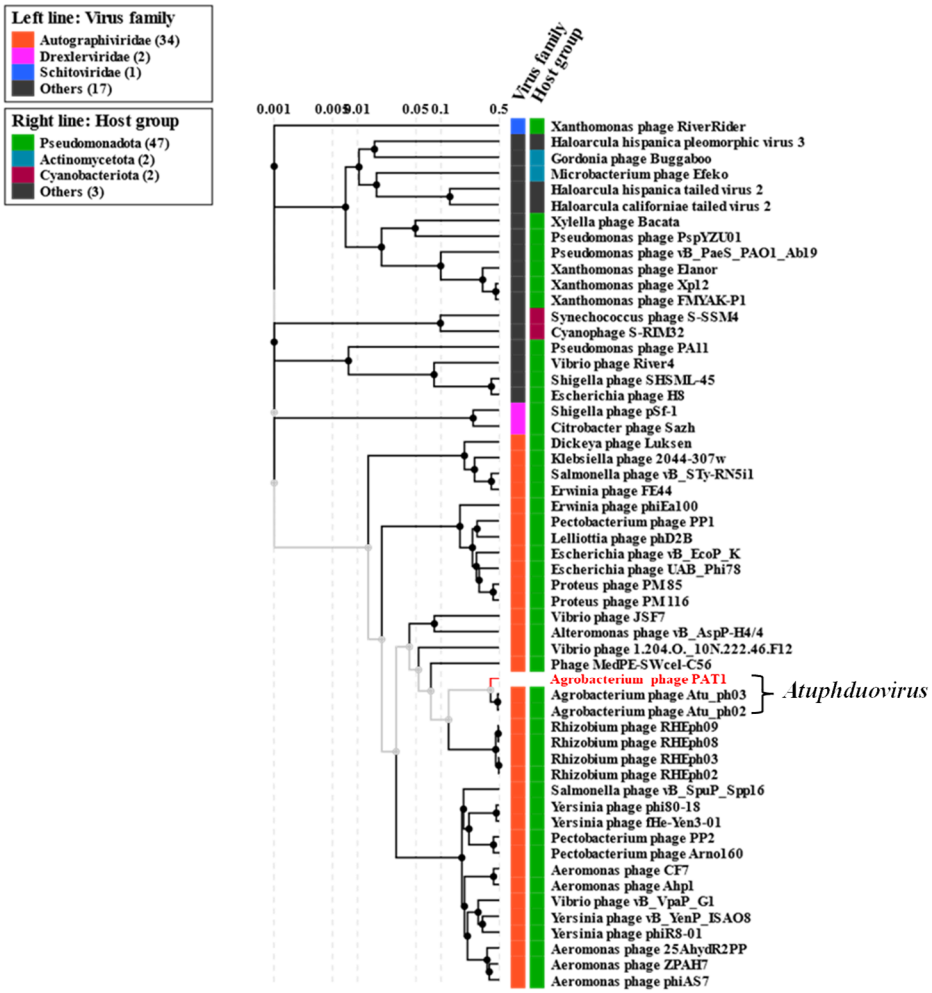

Figure 8. Proteomic tree of PAT1, generated by ViPTree based on genome-wide sequence similarities computed by tBLASTx, showing the allocation of PAT1 among species belonging to the genus *Atuphduovirus* within the family *Autographiviridae*.

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4. Discussion

Currently, phages are regarded as one of the most efficient biocontrol agents due to their unique characteristics, including their high selectivity in targeting specific bacteria without disrupting beneficial microbiota, hence maintaining environmental balance [23]. Additionally, phages self-replicate at the infection site, minimizing the need for repeated applications and ensuring sustained antibacterial activity [24]. Moreover, unlike other biocontrol agents, where the development of resistance is often irreversible, bacteriophages have the unique ability to adapt and evolve alongside bacterial populations, by evolving novel mechanisms to counteract this resistance ensuring sustained efficacy in biocontrol applications [25]. However, the scarcity of effective lytic phages against *A. tumefaciens* underscores a critical gap in the biocontrol arsenal [18]. Addressing this deficiency is crucial, as *A. tumefaciens* poses significant threats to agricultural productivity. With this aim, this study reports the isolation and characterization of a novel potent lytic phage, *i.e.*, phage PAT1, thereby enriching the available pool of *A. tumefaciens* phages and providing a targeted, eco-friendly solution to manage this dangerous bacterial pathogen.

Wastewater treatment stations normally collect sewage from various sources such as farms, hospitals, industry, and others. Thus, these stations could host a diverse range of bacterial communities, making it an ideal habitat for bacteriophage isolation. In this context, a lytic phage against *A. tumefaciens*, named *Agrobacterium* phage PAT1, was isolated and characterized from the untreated influx point at the wastewater processing station of Bari, Italy. TEM analysis showed that PAT1 displays morphological characteristics similar to those of podoviruses classified under the taxonomic class *Caudoviricetes*, while genomic and phylogenetic analyses have further identified PAT1 as a novel species within the genus *Atuphduovirus* of the family *Autographiviridae*. In assessing the suitability of PAT1 as a biocontrol agent, prediction of genes functions in the PAT1 genome showed the absence of known genes associated with antibiotic resistance, lysogenic, toxins or other virulence factors. Additionally, PAT1 was found to maintain its stability over a wide range of pH (4-10) and temperatures (4 °C to 60 °C). Host range analysis revealed that PAT1 is highly specific in its host range, with the ability to lyse only two strains of *A. tumefaciens* out of six examined. PAT1 was also inactive against other bacterial species tested, indicating that PAT1 is likely specific to certain strains of *A. tumefaciens*. This host range is comparable to that of previously described *A. tumefaciens*-infecting phages (i.e., *Agrobacterium tumefaciens* phages Atu_ph04 and Atu_ph08), which were shown to be unable to infect certain *A. tumefaciens* strains [18]. The narrow host range can be advantageous in PAT1 applications as it potentially cannot infect non-target beneficial bacteria, providing precise disease control. However, the high specificity of PAT1 for limited strains of *A. tumefaciens* can hinder its effectiveness for use in biocontrol of crown gall disease. To address this shortcoming, phage engineering offers a mean to extend the host range of bacteriophages [26]. Gene editing techniques such as the CRISPR-Cas system are used to replace or modify receptor binding proteins (RBPs) to recognize new hosts, thereby augmenting the spectrum of strains targeted by the engineered phage [26,27]. Moreover, it is now possible to strategically change the host range of bacteriophages by using advanced high-throughput methods like transposon sequencing and iCRISPR technology to identify specific bacteriophage receptor recognition genes and then introducing modifications or performing gene swapping through in-host recombination or out-of-host synthesis [26].

The lytic activity of PAT1 against *A. tumefaciens* was examined through a series of assays and microscopy analyses. The results of TEM analysis demonstrated the ability of PAT1 to complete its lytic life cycle on *A. tumefaciens* cells within 30 min. Results from the killing curve assay showed that PAT1 significantly inhibited the bacterial growth of *A. tumefaciens* for 24h, with the highest MOI showing the highest reduction value. However, further incubation to 48h resulted in an increase in the OD reading in both control and phage treated samples, which reflect the emergence of phage-resistant mutants. Even at this stage, the growth of *A. tumefaciens* treated with PAT1 (MOI = 1) was reduced by 82% compared to untreated bacteria.

The phenomenon of phage resistance (bacterial resistance to phages) is mediated through a variety of adaptive mechanisms, including alterations in surface receptor structures that prevent phage adsorption, bacterial capsule modifications, and the activation of intrinsic bacterial defense

 doi:10.20944/preprints202407.2228.v1

systems such as CRISPR-Cas [28]. Such resistance mechanisms are not uncommon and have been observed across a broad spectrum of bacterial species [29]. To deal with this resistance, previous studies demonstrated that the combination of phages with other antimicrobial compounds (*i.e.*, bacteriocins, antimicrobial peptides, antagonistic bacteria) leverages the specific targeting abilities of phages and the diverse mechanisms of other antimicrobial agents, leading to enhanced bacterial control reaction and reduced risk of resistance development [30]. Therefore, employing PAT1 in conjunction with other antimicrobial compounds may help target bacteria more effectively, preserving the therapeutic potential of PAT1 and reducing the risk of resistance development. Furthermore, bacteriophage-derived endolysins, which are recognized as powerful and broad bactericidal agents that can rapidly and precisely hydrolyze bacterial cell walls, are gaining increasing interest as potential alternatives to antibiotics [31–33]. These phage-encoded enzymes have been shown to exert bactericidal activity both individually and synergistically when combined with other antibacterials, thereby enhancing their efficacy [34]. For example, the combination of the phage endolysin SAL200 with SOC antistaphylococcal antibiotics showed synergistic effects in vitro and in vivo on *Staphylococcus aureus* infections [35]. Furthermore, endolysins have been employed successfully against plant pathogenic bacteria, indicating their promise in sustainable agriculture [36,37]. In our case, genomic analysis revealed the presence of two endolysins within PAT1 genome, which can also be exploited against *A. tumefaciens*, highlighting the possibility of using endolysins from our phage, alone or in conjunction with other antimicrobials, to develop an integrated and effective biocontrol strategy against crown gall disease.

5. Conclusions

In conclusion, the novel characterized bacteriophage demonstrated several advantageous properties, namely high stability at a wide range of pH and temperatures, absence of toxin, lysogenic or antibiotic resistance genes in its genome, rapid infectious cycle, presence of two endolysins genes, and robust lysis potential against *A. tumefaciens*. These attributes position PAT1 as a potential agent for controlling crown gall disease or as a component of an integrated management strategy. However, further investigations are required to explore the *in-planta* efficacy of PAT1 individually and in combination with other antibacterials.

Author Contributions: M.S.: writing—original draft, visualization, validation, software, methodology, investigation, formal analysis, data curation, and conceptualization. K.E.H.: writing—original draft, visualization, validation, software, methodology, investigation, formal analysis, data curation, and conceptualization. O.C.: software, investigation, and formal analysis. A.D.S.: writing—review and editing, methodology, investigation, conceptualization, and validation. T.E.: writing—review and editing, visualization, supervision, validation, software, methodology, formal analysis, data curation, conceptualization, resources, project administration, and funding acquisition. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: No ethics approval was required for this work.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: The authors thank Dr. Franco Valentini from CIHEAM Bari (IPM Department) and Dr. Maria Holeva from Benaki Phytopathological Institute, BPI (Phytopathology Department, laboratory of bacteriology) for generously providing us with the bacterial strains used in this study. Their support and contributions were invaluable to the successful completion of our research.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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