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Isolation, characterization and genomic analysis of a novel lytic bacteriophage infecting *Agrobacterium tumefaciens*

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Summary. *Agrobacterium tumefaciens* causes crown gall, and economic losses in important crops, including apple, pear, peach, and almond. Difficulties controlling this disease with conventional pesticides require alternative antibacterial agents. A novel lytic bacteriophage, *Agrobacterium* phage PAT1 (PAT1), with high lysis potential against *A. tumefaciens*, was isolated from wastewater. Interaction between PAT1 and *A. tumefaciens* cells was investigated using transmission electron microscopy. PAT1 adsorbed, infected, and replicated on *A. tumefaciens* in ≤30 min. Turbidity assays showed that PAT1 [Multiplicity of Infection (MOI) = 1] inhibited *A. tumefaciens* growth by 82% for 48 hours. PAT1 was resistant to broad ranges of pH (4 to 10) and temperatures (4 to 60°C). Bioinformatics analyses of the PAT1 genomic sequence showed that the bacteriophage was closely related to *Atuphduovirus* (*Autographiviridae*) phages. The PAT1 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 23 CDS were predicted, including an endolysin gene which could be used as an antimicrobial against *A. tumefaciens*. No lysogenic mediated genes or genes encoding virulence factors, antibiotic resistance, or toxins were detected in PAT1 genome. The bacteriophage showed potential as a biocontrol agent against *A. tumefaciens* infections, expanding the limited catalogue of lytic *A. tumefaciens* phages, although efficacy for control of crown gall *in planta* remains to be evaluated.

Keywords. Plant pathogenic bacteria, crown gall, phage therapy, biocontrol.

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INTRODUCTION

Agrobacterium tumefaciens is a Gram-negative, non-spore-forming, motile, rod-shaped plant pathogenic bacterium that causes crown gall many

plant species (Etminani *et al.*, 2022). Crown gall compromises commercialization of plants in more than 60 families, including dicotyledonous plants, ornamentals brambles, and pome fruit, stone fruit, and nut trees (Lee *et al.*, 2009; Choi *et al.*, 2019; Etminani *et al.*, 2022). *Agrobacterium tumefaciens* is commonly found in the rhizospheres of many plants, where it survives on root exudates (Eckardt, 2006). The bacterium infects host plants through wounds roots, stems and crowns, which often occur in orchards during pruning and in nurseries through transplanting and grafting (Eckardt, 2006; Etminani *et al.*, 2022). The pathogen then becomes pathogenic by transforming plants with a fragment of the tumor-inducing (Ti) plasmid, a transfer-DNA (T-DNA) which induces abnormal proliferation of host plant cells via synthesis of phytohormones, leading to the formation of tumours (galls) (Kawaguchi *et al.*, 2019; Thompson *et al.*, 2020). Galls usually develop at plant crowns but can also occur above ground on secondary or lateral roots and main stems. These tumours restrict water and nutrient flow causing yield losses and, in severe cases, plant death (Eckardt, 2006; Asghari *et al.*, 2020).

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* isolate K84 and its genetically modified isolate K1026, have been shown to be effective in several locations (Penyalver *et al.*, 2000). However, K84 and K1026 are ineffective against some strains of *A. tumefaciens*; thereby limiting their ability to provide broad-spectrum control (Vicedo *et al.*, 1993). Therefore, there is a requirement to identify new effective biocontrol agents against *A. tumefaciens*.

Virulent (lytic) bacteriophages, which are viruses that specifically infect and lyse bacteria, are potential options for field scale biological control. These bacteriophages are ubiquitous, recognized as safe agents, and are potent antibacterial agents in agriculture (Svircev *et al.*, 2018; Álvarez *et al.*, 2019; Sabri *et al.*, 2022). Lytic phages have advantageous characteristics, including ease of discovery, high host bacterium specificity, self-replicating nature, harmlessness to eukaryotes, low environmental impacts, low cost and simplicity for preparation, high efficiency at low multiplicity of infection (MOI). Their post-application levels increase reducing bacterial host survival, in contrast with antimicrobial compounds (Loc-Carrillo and Abedon, 2011; Sabri *et al.*, 2024).

Seven lytic phages have been reported to infect this bacterial plant pathogen. These are: 7-7-1 (Kropinski *et al.*, 2012), Atu_ph02 and Atu_ph03 (Attai *et al.*, 2017), Atu_ph07—a jumbo phage (Attai *et al.*, 2018), Atu_ph04 and Atu_ph08 (Attai and Brown, 2019), and Milano (Nittolo *et al.*, 2019). The present study isolated and characterized a novel lytic phage of *A. tumefaciens,* named *Agrobacterium* phage PAT1 (PAT1), which demonstrated *in vitro* antibacterial efficacy against *A. tumefaciens*.

MATERIALS AND METHODS

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^{-1} yeast extract, 5.0 g L^{-1} peptone, 10.0 g L^{-1} glucose) or on yeast extract peptone glucose agar (YPGA, YPG supplemented with 1.5% agar).

Bacteriophage isolation, purification, and titration

The phage described in the present study was isolated from a sewage water sample collected in April 2023 at the untreated influx point of the wastewater processing station in Bari (south of Italy; Latitude: 41.1081° N, Longitude: 16.2606° E). One L of sewage water was passed through a 75 × 100 mm Grade 1 filter paper (Whatman) to remove large particles, and the filtered through a 0.22 µm filter (Merck) to remove cellular debris. The resulting filtrate was centrifuged at 109,000 (Rotor J50.2 Ti, Beckmann Coulter) for 1 h at 4°C to pellet phage particles. The pellet was resuspended in 2 mL of 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 for 24 h and transferred to 2 mL of YPG broth at 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 24 h. Phage was purified from filtrate using the double agar overlay method (Kropinski *et al.*, 2009). A single clear plaque-forming unit was transferred into 1 mL of phage buffer and this process was repeated three times to ensure isolation of a single phage. To obtain high phage titre, 1 mL of *A. tumefaciens* strain CFBP 5770 culture at OD 600 of 0.2 was inoculated into 500 mL of YPG broth, 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 (108,800 g for 1 h), resuspended in 2 mL of phage buffer, and stored at 4°C for further analysis. The phage titre was determined through a double-layer assay.

Table 1. Bacterial isolates used for determining the host range of PAT1.

Species	Isolate[a]	Host plant	Origin
Xanthomonas campestris pv. *campestris*	CFBP 1710	*Brassica oleracea* var. *botrytis*	France
Xanthomonas albilineans	CFBP 1943	-	Burkina Faso
Erwinia amylovora	PGL Z1 [b]	*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

[a] CFBP: French Collection of Phytopathogenic Bacteria, Angers, France. YD: Collection of bacterial strains isolated in diagnostic work of the bacteriology laboratory, Benaki Phytopathological Institute. BPIC: Benaki Phytopathological Institute collections.
[b] Collection of CIHEAM-IAM, Bari, Italy.

Spot assays of phage lytic activity

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^{-1}) were mixed with soft agar (YPG supplemented with 0.7% agar), which was poured into a petri plates (6 mL per plate) and allowed to dry. Drops (10 µL each) of phage solution containing 108, 107, 106, 105, or 104 PFU mL^{-1} were spotted onto the surfaces of the plates. The spots were dried at room temperature and the plates then cultured for 24 h at 28°C.

Transmission Electron Microscopy (TEM)

To assess the morphological and lytic properties of the purified phage PAT1, a culture of *A. tumefaciens* strain CFBP 5770 was challenged with PAT1 (MOI = 1) for 1 h at room temperature. Representative images of the phage and bacterium cells were taken at 10-, 30-, or 60-min intervals post-infection (pi) using a transmission election microscope (FEI MORGAGNI 282D) using the dip method. Carbon-coated copper/rhodium grids underwent 2 min. incubation with either the phage alone or with 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). The samples were viewed at microscope accelerating voltage of 80 kV.

DNA extraction, whole-genome sequencing, and bioinformatic analysis of PAT1

Genomic DNA of PAT1 was extracted from a high-titre stock of phage particles at ~10^{10} PFU mL^{-1} using a DNeasy Plant Extraction kit, following the manufacturer's protocol (Qiagen). The extracted DNA was quantified using a NanoDrop™ One/OneC Microvolume UV-Vis Spectrophotometer (ThermoFisher Scientific). Subsequently, 500 ng of purified genomic DNA was sent for Illumina sequencing (2 × 150 bp paired-end mode) (Eurofins Genomics). The reads were quality checked and trimmed using BBDuk Trimmer 1.0 and *de novo* assembled using the Tadpole tool with different k-mers (Geneious Prime 2024.0.7). The nucleotide sequence similarity of the obtained phage with those reported in GenBank was calculated based on a complete nucleotide alignment of the genomic sequences of most PAT1-related phages using Geneious. The functions of ORFs were annotated with Geneious, using the complete genomic sequences of the phages most closely related at the molecular level to PAT1, i.e., *Agrobacterium* phage Atu_ph02 (accession number NC_047845) and *Agrobacterium* phage Atu_ph03 (accession number NC_047846); and the HHpred and HHblits of the Bioinformatics

toolkit (https://toolkit.tuebingen.mpg.de/tools/hhpred). A search for tRNA genes in the genome of PAT1 was performed using Prokka 1.14.0 (https://Kbase.us).

The predictions of antibiotic resistance genes, acquired virulence genes, and toxin-encoding genes were assessed using, respectively, resFinder 4.5.0, VirulenceFinder 2.0.5 and ToxFinder 1.0 softwares, in the CGE tool (http://www.genomicepidemiology.org/). The complete genome sequence of PAT1 was deposited at GenBank and a circular map of the genome and phylogenetic tree were constructed using g ViPTree (Nishimura *et al.*, 2017).

Optimal multiplicity of infection (MOI) of phage PAT1

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

Host range analysis

The host range of PAT1 was assessed using the phage sensitivity spot test described above. Bacterial strains listed in Table 1 were cultured at 28°C on YPGA plates for up to 2 d. The cultures were then each suspended in sterile distilled water, and 200 µL of bacterial suspension (OD600 = 0.2) were mixed with 6 mL volumes of YPG soft agar, which were then poured into Petri plates, and allowed to dry. Drops (10 µL each) of phage solution at 10^8, 10^7, 10^6, 10^5, or 10^4 PFU mL^{-1} were spotted onto the surfaces of the plates, which were then dried at room temperature. The plates were then incubated for up to 2 d at 28°C. Presence of clear zones was recorded for the respective strains, indicating strain susceptibility to PAT1.

Temperature and pH effects on PAT1

Temperature effects on PAT1 were assessed by incubating 100 µL of phage suspensions (~10^7 PFU mL^{-1}) for 1 h, at 4, 28, 40, 50, 60, or 70°C. Following incubation, serial dilutions were made with phage buffer, and phage titres were determined using the double agar overlay method. To assess pH effects, phage suspensions (100 µL) were each to 900 µL of sterile-filtered YPG that was pH-adjusted using 1 M NaOH or 1 M HCl. The inoculated cultures were then incubated at 28°C for 1 h. Subsequently, serial dilutions were made with phage buffer, and phage titres were determined using the double agar overlay method.

RESULTS

Spot assays

PAT1 produced clear lysis zones on the *A. tumefaciens* lawns (Figure 1) at all assessed titres, demonstrating the lysis potential of PAT1 against the bacterium.

Morphological and lytic properties of PAT1

PAT1 produced clear plaques, ranging from 2 to 5 mm diam. on lawn cultures of the *A. tumefaciens* strain CFBP 5770 (Figure 2A). TEM analysis showed that PAT1 had morphological features typical of a podovirus morphotype C1, with icosahedral and Head-tail geometries. The capsid diameter was approx. 60 (± 3) nm (length/width ratio = 1), and the non-contractile tails were 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 adsorption

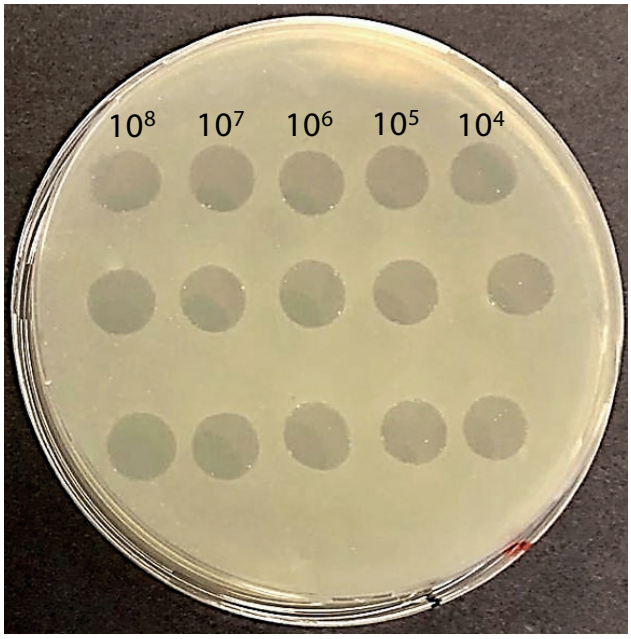

Figure 1. A YPGA plate showing antibacterial activity against *Agrobacterium tumefaciens* of PAT1 at different titres (10^8 to 10^4 PFU mL^{-1}, in triplicates).

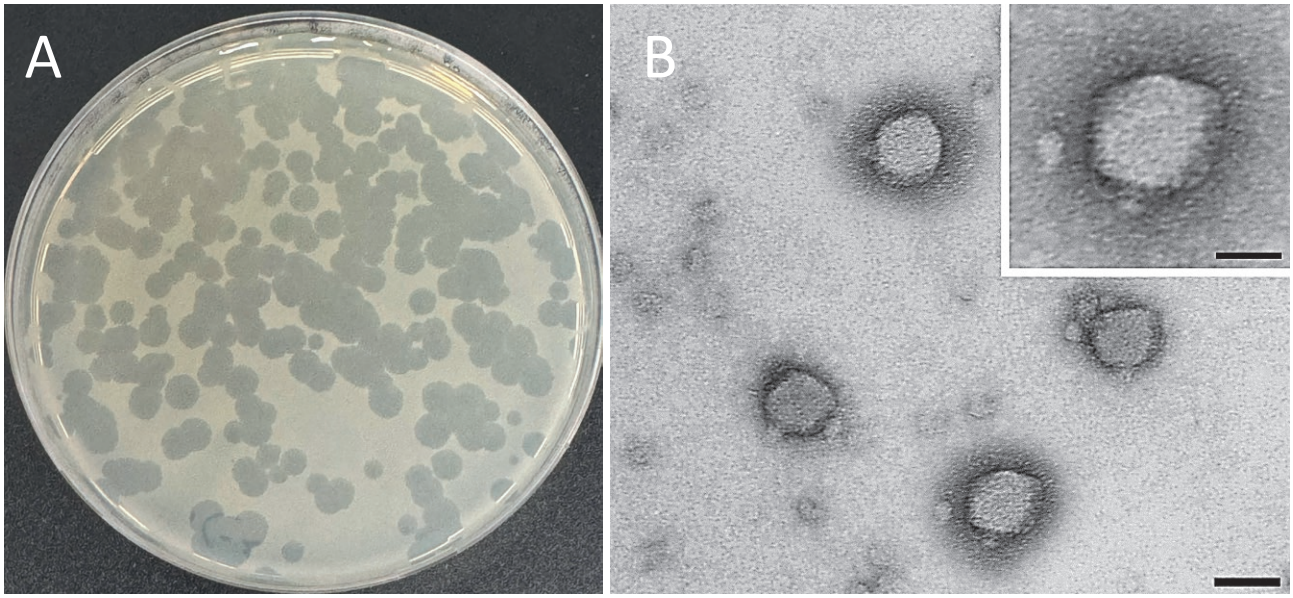

Figure 2. (A) Plaques caused by PAT1 on an *Agrobacterium tumefaciens* double layer agar plate. (B) Transmission electron microscope image of PAT1 showing a particle each with an icosahedral capsid and a very short non-contractile tail. Scale bars: 50 nm and 25 nm (inset).

of PAT1 on cell surfaces of *A. tumefaciens* at 10 min pi (Figure 3B), while the lysed cells of *A. tumefaciens* and release of progeny virions from infected bacteria were visualized at 30 min pi (Figure 3, C and D). These observations demonstrated the ability of PAT1 to adsorb, replicate and kill *A. tumefaciens* in less than 30 min for a complete infection cycle, and indicate that the infection cycle was lytic.

Temperature and pH effects on phage PAT1, and host range

The thermal and pH effects on PAT1 were estimated by measuring variations in survival rates as functions of the numbers of plaque-forming units (PFU). The phage was generally stable at temperatures from 4°C to 60°C, while it had approx. tenfold less infectivity at 60°C, and incubation at 70°C for 1 h killed the phage (Figure 4 A). PAT1 also had stable infectivity across the assessed pH range of 4 to 10 (Figure 4 B).

Host range analysis of PAT1 carried out for 16 bacterial strains (Table 1) showed that strains CFBP 5770 and BPIC 284 of *A. tumefaciens* were equally susceptible to PAT1, with the phage producing clear lysis zones at the different tested titres (data not shown). However, PAT1 was inactive against other *A. tumefaciens* strains, and the plant pathogenic bacteria examined, indicating that this phage is likely to be specific to strains of *A. tumefaciens*.

Bacteriolytic effects of PAT1 on growth of Agrobacterium tumefaciens

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 effective, and the phage restricted growth of *A. tumefaciens* for 24 h (Figure 5). However, after 24 h, PAT1-infected bacteria displayed increased ODs, indicating emergence of phage-resistant mutants. Additionally, bacteria treated with the greatest MOI (1) showed slower increase in OD readings after 24 h (Figure 5), so MOI = 1 was determined as optimal MOI for PAT1. Despite the resistance development, PAT1 (MOI = 1) gave considerable antibacterial activity against *A. tumefaciens* growth, achieving an 82% reduction in bacterial growth at 48 h pi. At the end of the experiments, PAT1-resistant mutants were isolated on YPGA agar plates, and resistance to PAT1 was confirmed by spot assay. These results show that PAT1 possesses effective inhibitory potency against *A. tumefaciens*, indicating its potential for controlling crown gall.

Genomic and phylogenetic analyses of PAT1

The whole genome sequencing and *de novo* assembly of PAT1 revealed a double-stranded DNA genome of length of 45,040 base pairs, with a G + C content of

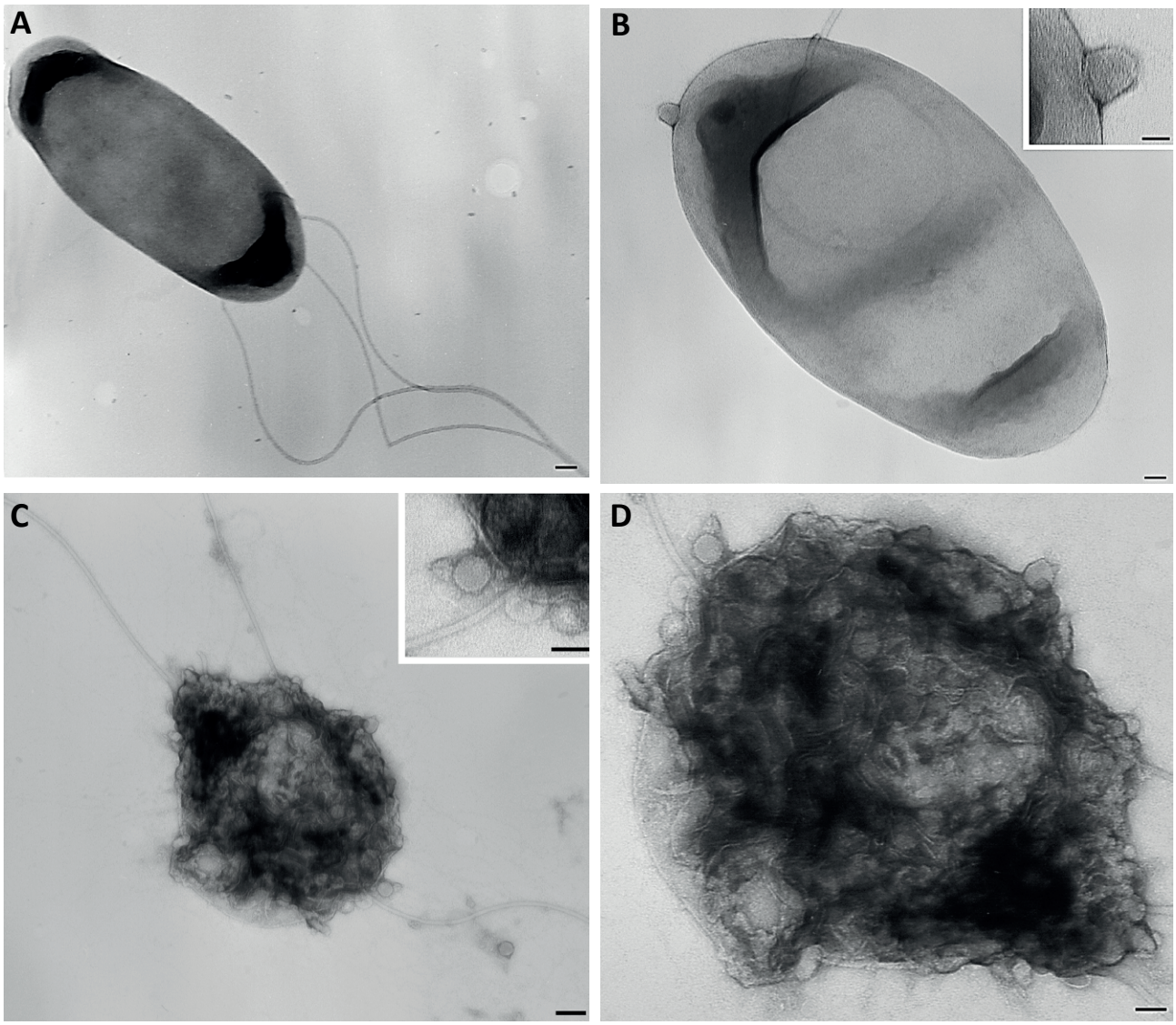

Figure 3. Transmission electron micrographs of *Agrobacterium tumefaciens* cells challenged with PAT1. A. untreated *tumefaciens* cell, used as control. B. PAT1 attachment on an *A. tumefaciens* cell surface (inset shows the point of phage penetration). C and D. Lysis of PAT1-treated *A. tumefaciens*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

54.5%. This is less than that of *A. tumefaciens* (average 58.5%) (Deschamps *et al*., 2016). The complete genome of PAT1 consisted of 54 coding sequences (CDSs), of which 31 (57.4%) encode for hypothetical proteins. The functions of 23 CDSs (42.6%) were predicted. These 23 CDs encode proteins involved with DNA replication and regulation, DNA packaging and structural proteins, and cell lysis, as highlighted on the genomic map (Figure 6). Prokka and Geneious analyses showed no tRNA encoding genes were present in the genome of PAT1. CGE analysis showed that the PAT1 genome did not contain any known genes associated with antibiotic resistance, lysogenicity, toxins, or other virulence factors. These results indicate that PAT1 is suitable for use as a biocontrol agent.

Genome sequence analysis also showed that PAT1 shared maximum nucleotide similarities of 78.7% with Agrobacterium phage Atu_ph02 (accession number NC_047845) and 78.5% with Agrobacterium phage Atu_ph03 (accession number NC_047846) (Figure 7). Both phages are members of *Atuphduovirus* (*Autographiviridae*) and are known to infect *A. tumefaciens*. Based on the demarcation criteria of the "International Committee on Taxonomy of Viruses" (ICTV) for classification

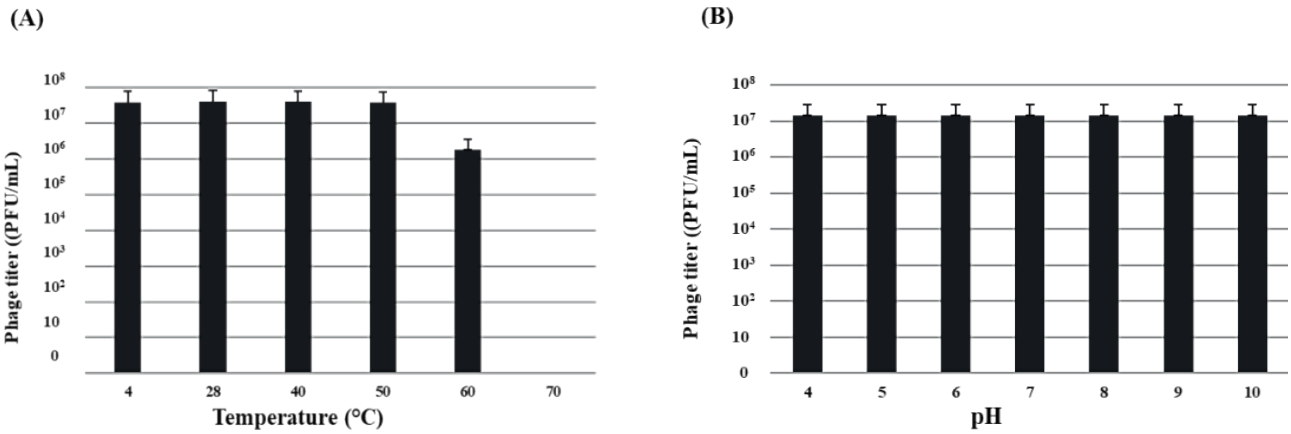

Figure 4. Histograms showing results of thermal and pH stability tests of PAT1. (A) Phage titre after being treated with different temperatures for 60 min. (B) Phage infectivity after incubation at different pHs for 60 min. Phage titres were determined using the double agar overlay method. Error bars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

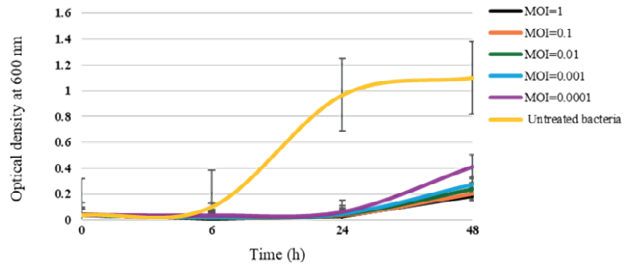

Figure 5. Growth curves for *Agrobacterium tumefaciens* treated with PAT1 at different MOIs. Mean optical densities of the bacterium cultures are shown for untreated bacteria or after different MOI treatments, up to 48 h pi. The bars indicate standard errors of the means for three replicates.

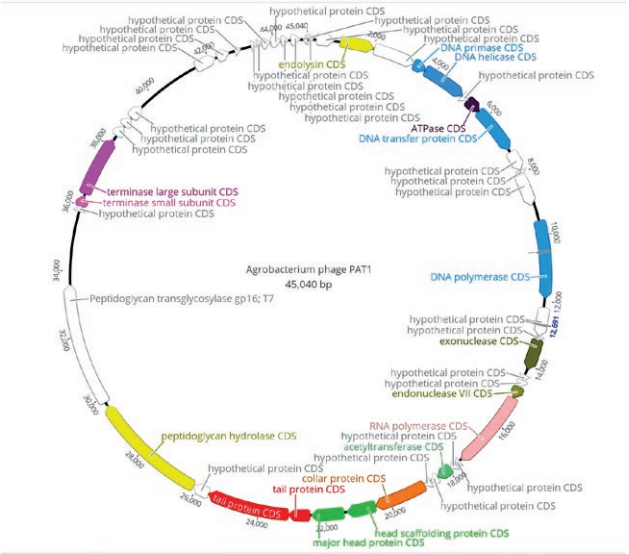

Figure 6. Genomic map of PAT1, representing 54 coding sequences encoded by the genome. Hypothetical proteins are displayed in grey, and predicted proteins are indicated with assigned functions highlighted with different colours.

of new bacteriophages species (sequence similarity ≤ 95%), PAT1 is a putative new species, and is accordingly named Agrobacterium phage PAT1.

The compete genome sequence of PAT1 was deposited in GenBank under the accession number PQ082932. The proteomic tree of the 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, in *Atuphduovirus* in *Autographiviridae* (Figure 8). Therefore, PAT1 is considered as a tentative novel member of *Atuphduovirus*.

DISCUSSION

Phages are currently regarded as efficient biocontrol agents, due to their characteristics of high selectivity in targeting specific bacteria without disrupting beneficial microbiota, hence maintaining environmental balance (Federici *et al*., 2021). Additionally, phages self-replicate at infection sites, minimizing the need for repeated applications and ensuring sustained antibacterial activity (Xu *et al*., 2022). Unlike other biocontrol agents, where development of resistance is often irreversible, bacteriophages have the unique ability to adapt and evolve alongside bacterial populations, by developing novel mechanisms to counteract this resistance to ensure sustained efficacy of biocontrol applications (Borges, 2021). However,

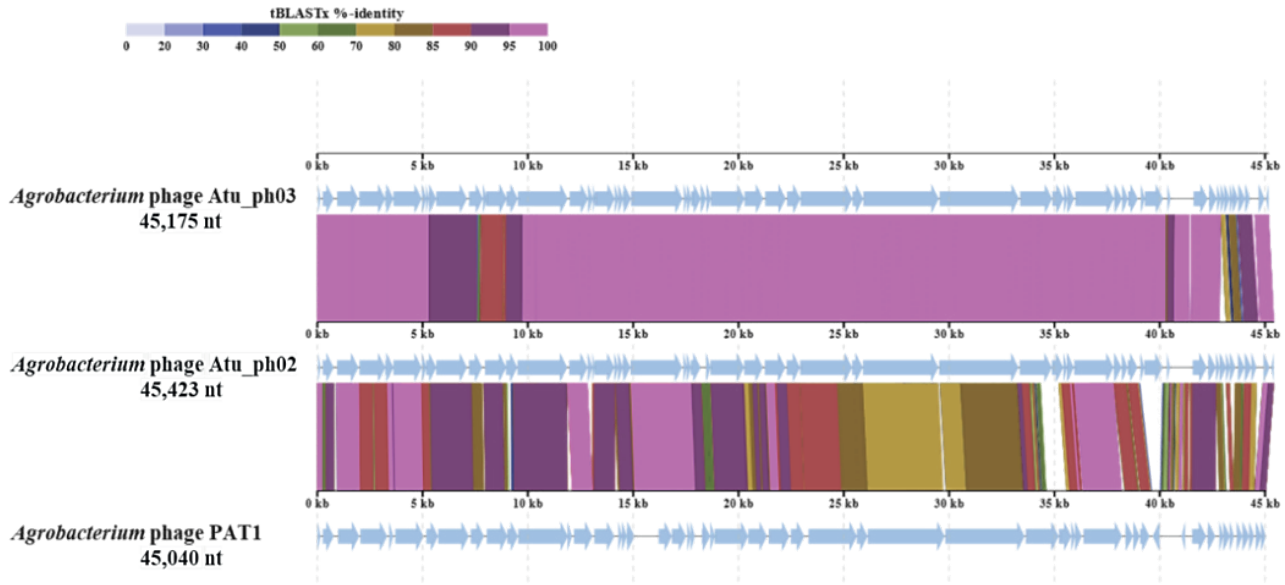

Figure 7. Genomic alignment of PAT1 with its close homologues. The coloured vertical blocks between the genomes indicate levels of nucleotide similarity. The genome alignment was generated using ViPTree.

the scarcity of effective lytic phages against *A. tumefaciens* underscores a critical gap in the biocontrol arsenal (Attai and Brown, 2019). Addressing this deficiency is important, as *A. tumefaciens* poses threats to agricultural productivity. The present study has described isolation and characterization of a novel and potent lytic phage, designated phage PAT1, thereby increasing the pool of *A. tumefaciens* phages, and providing a potentially eco-friendly solution for managing this plant pathogen.

Wastewater treatment stations normally collect sewage from many sources, such as farms, hospitals, industry, and elsewhere. These stations could be sources of a diverse range of bacterial communities, making them ideal habitats 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 had morphological characteristics like those of podoviruses in *Caudoviricetes*, while genomic and phylogenetic analyses further identified PAT1 as a novel species within *Atuphduovirus* (*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, lysogenicity, toxins or other virulence factors.

PAT1 was found to maintain stability of activity over a wide range of pH (4 to 10) and temperature (4°C to 60°C). Host range analysis showed that PAT1 is host-specific, 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 to be specific to 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 A. *tumefaciens* strains (Attai and Brown, 2019). The narrow host range can be advantageous in PAT1 applications, as the phage potentially cannot infect non-target beneficial bacteria, likely to provide 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 could host ranges of bacteriophages (Jia *et al*., 2023). Gene editing techniques such as the CRISPR-Cas system can replace or modify receptor binding proteins (RBPs) to allow phages to recognize new hosts, thereby augmenting spectra of strains targeted by engineered phages (Jia *et al*., 2023; Gencay *et al*., 2024). It is also possible to strategically change the host range of bacteriophages, using advanced high-throughput methods such as transposon sequencing and iCRISPR technology, to identify specific bacteriophage receptor recognition genes, and then introducing modifications or performing gene swapping through in-host recombinations or out-of-host syntheses (Jia *et al*., 2023).

The lytic activity of PAT1 against *A. tumefaciens* was examined through a series of assays and microscopy analyses. The results of TEM analysis demonstrated

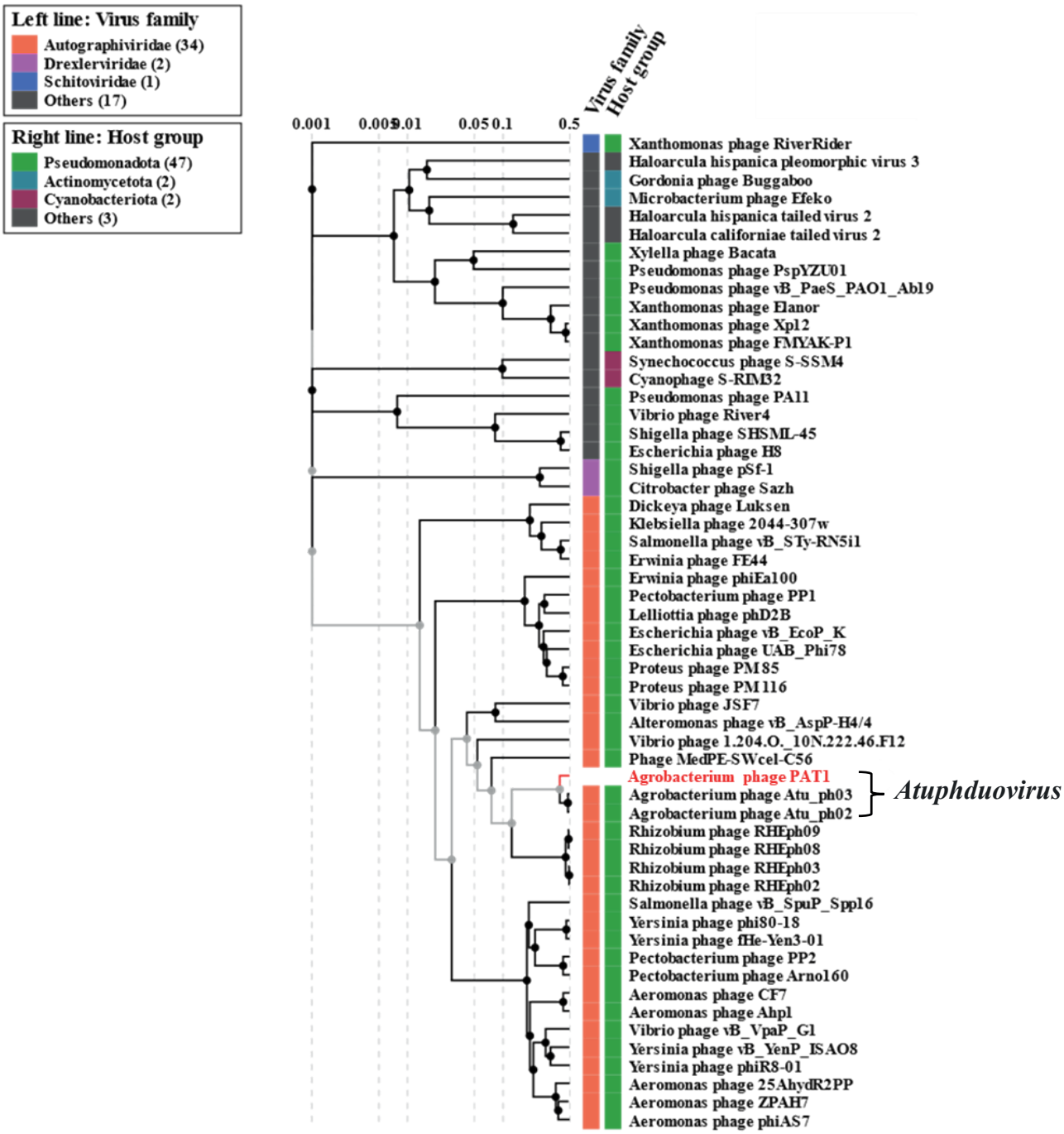

Figure 8. Proteomic tree of PAT1, generated by ViPTree based on genome-wide sequence similarities computed by tBLASTx, showing the allocation of PAT1 among species belonging to *Atuphduovirus* (*Autographiviridae*).

the ability of PAT1 to complete its lytic life cycle in *A. tumefaciens* cells within 30 min. Results from the MOI assays showed that PAT1 inhibited growth of *A. tumefaciens* for 24 h, with the greatest MOI giving the greatest reductions. However, incubation to 48 h resulted in increased ODs, both in control and phage treated samples, which reflect emergence of phage-resistant mutants. At this time stage, however, growth of *A. tumefaciens* treated with PAT1 (MOI = 1) was still reduced by 82% compared to untreated bacteria.

Phage resistance in bacteria is mediated through several adaptive mechanisms, including alterations in surface receptor structures that prevent phage adsorption, bacterial capsule modifications, and activation of intrinsic bacterial defense systems such as CRISPR-Cas (Egido *et al*., 2022). These resistance mechanisms have been detected observed across several bacterial species (Hyman and Abedon, 2010). To deal with this resistance, previous studies demonstrated that combinations of phages with other antimicrobial compounds (i.e., bacteriocins, antimicrobial peptides, antagonistic bacteria) leverages specific targeting abilities of phages and the mechanisms of other antimicrobial agents, leading to enhanced bacterial control reactions and reduced risks of resistance development (Knezevic and Aleksic Sabo, 2019). Therefore, employing PAT1 in conjunction with other antimicrobial compounds may increase efficiency of anti-bacterial activity, preserving the therapeutic potential of PAT1 and reducing the risk of resistance development. Furthermore, bacteriophage-derived endolysins, which are recognized as powerful and broad-spectrum bactericidal agents that can rapidly and precisely hydrolyze bacterial cell walls, are potential alternatives to antibiotics (Wong *et al*., 2022; Liu *et al*., 2023; Khan *et al*., 2024). These phage-encoded enzymes exert bactericidal activity both individually, and synergistically when combined with other antibacterials, thereby enhancing their efficacy (Fischetti, 2018). For example, the combination of the phage endolysin SAL200 with SOC anti-staphylococcal antibiotics gave synergistic effects *in vitro* and *in vivo* on *Staphylococcus aureus* infections (Kim *et al*., 2018). Endolysins have also been employed successfully against plant pathogenic bacteria, indicating their promise in sustainable agriculture (Vu and Oh, 2020; Nazir *et al*., 2023). In the present study, genomic analysis revealed the presence of an endolysin within the PAT1 genome, which could also be exploited against *A. tumefaciens*. This highlights the possible use of endolysin from PAT1, alone or in conjunction with other antimicrobials, to develop an integrated and effective biocontrol strategy against crown gall.

Phage PAT1 is a new biological agent in the list of phages that have been reported to specifically target *Agrobacterium* spp., particularly those responsible for plant diseases. On a practical level, these phages have shown potential for applications in agriculture. For example, the OLIVR1 phage has been successfully used to disinfect hydroponic greenhouse nutrient solutions from *A. rhizogenes*, the pathogen responsible for hairy roots in greenhouse-grown plants (Fortuna *et al*., 2023). The research in the present study was limited to the identification and characterization of PAT1, as an initial step towards evaluating its lysis potential and suitability for combating *A. tumefaciens*. The results obtained position PAT1 as a promising candidate for further evaluation in combating *A. tumefaciens* infections, both in greenhouse and in the field horticulture and agriculture.

CONCLUSIONS

The bacteriophage characterized in this study has several advantageous properties, including high stability over wide pH and temperature ranges, absence of toxins, lysogenicity or antibiotic resistance genes, a rapid infections cycle, presence of two endolysins genes, and good lysis potential against *A. tumefaciens*. These attributes indicate that PAT1 has potential for controlling crown gall, or as a component of integrated management of this disease. However, further investigations are required to explore the *in-planta* efficacy of PAT1, and its combination with other antibacterial agents.

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AUTHOR CONTRIBUTIONS

M.S. and 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 the authors have read and agreed to the published version of the manuscript.

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