

Presentation of Three Novel Tailed Phages Targeting Multiple Strains of *Pseudomonas syringae*

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Abstract

Background: *Pseudomonas syringae* are ubiquitous epiphytic plant pathogens infecting a wide range of important agricultural plant species. Bacteriophages has been proposed as biocontrol agents against plant pathogens, however, in order to utilize this approach, a deeper understanding of phage diversity and phage-host interactions is required.

Materials and Methods: Phages targeting *P. syringae* GAW0113 were isolated from organic waste samples. Three distinct phage isolates were purified and subjected to whole-genome sequencing, comparative genomics, transmission electron microscopy and host-range assay using a wide selection of diverse *P. syringae* isolates.

Results: The three phage isolates, *Pseudomonas* phage Bertil, Misse, and Strit, were shown to have podovirus morphology with a short tail stub and isometric head. They had double-stranded DNA ranging from 41,342 to 41,374 bp in size comprising 50–51 open reading frames. The three phage genomes were highly similar and genomic comparison analyses showed that they all belong to the *Autographiviridae* family of the order *Caudovirales*. All three phages were shown to have a narrow host-range.

Conclusions: The three phages were shown to share morphological and genomic features with other phages in the *Autographiviridae* family, however, based on the limited nucleotide similarity we propose that the phages constitute a novel genus. All three phages were found to infect multiple strains of *P. syringae* covering several phylogroups.

Keywords: bacteriophage, biocontrol, *Pseudomonas* phage, *Pseudomonas syringae*, phage therapy

Introduction

PSEUDOMONAS SYRINGAE is a gram-negative rod-shaped bacterium. Strains of *P. syringae* are ubiquitous epiphytic plant pathogens and can infect a wide range of important agricultural plant species. *P. syringae* has been known to cause diseases such as bacterial canker of wild cherries¹ and kiwifruit,² bacterial speck of tomato³ and leaf blight in wheat.⁴ Many *P. syringae* strains isolated from nonagricultural environments have functional type 3 secretion systems (T3SS).⁵ The T3SS has been linked to the virulence of *P. syringae*. The presence of strains with functional T3SS suggests that there is a reservoir of plant-pathogenic bacteria in other environmental niches than plants. The widespread presence of pathogenic *P. syringae* strains in agricultural plants calls for an inexpensive and sustainable method for preventing or treating bacterial infections. Bacteriophage therapy is a promising approach to disease management, but requires an extensive library of relevant phages and understanding of phage–host interaction between phages

and the *P. syringae* species.⁶ In this study, we present three phages isolated against *P. syringae* strain GAW0113: *Pseudomonas* phage Bertil, *Pseudomonas* phage Misse, and *Pseudomonas* phage Strit. No reports of phages isolated against GAW0113 were available at the time of this study. Even though GAW0113 has not been associated with disease in crops, characterization of novel phages targeting strains of *P. syringae* can help in understanding the diversity of *P. syringae* phages and phage–host interactions, including bacterial resistance mechanisms.⁷ Furthermore, the host-range assay of the three phages shows that they are able to target other strains within the *P. syringae* species complex. Increased diversity within the known phages targeting *P. syringae* strains also allow for development of more complex phage cocktails. One of the potential flaws of using phages as antimicrobial agents is that bacteria can develop resistance relatively quickly against specific phages.⁸ The use of therapeutic cocktails of diverse phages makes it harder for the target bacteria to adapt and for resistant cells to survive.⁶

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Materials and Methods

Isolation

Samples of primarily household organic waste (OW) from a waste management facility were collected, aliquoted, and filtered with 0.45 μm PVDF syringe filters (Merck Millipore, Darmstadt, Germany). The filtrates were tested for phage activity against *P. syringae* GAW0113 using a double agar overlay assay with 0.6% soft agarose top agar.⁹ To obtain pure isolates, plaques with distinct appearance or from different OW samples were collected with a sterile pipette tip and transferred to 900 μL SM buffer. The phage lysate was then diluted and replated using the same double agar method. This purification process was performed three times to obtain phage isolates from single plaques. The final phage isolates were stored at 4°C.

DNA extraction and sequencing

The phage DNA was extracted from lysate, as previously described.¹⁰ Five units of DNase (A&A Biotechnology, Gdynia, Poland) were added to 200 μL lysate and incubated at 37°C for 1 h followed by heat inactivation at 70°C for 10 min. Next, 6 U of Proteinase K (A&A Biotechnology, Gdynia, Poland) and sodium dodecyl sulfate–polyacrylamide [final concentration of 0.1% (w/v)] were added followed by another step of heat inactivation at 70°C for 10 min. The DNA solution was then purified with DNA Clean and Concentrator-5 kit (Zymo Research, California, USA). Phage DNA libraries were prepared using NEBNext Ultra II FS library Prep and Kit for Illumina (New England Biolabs, Ipswich, USA). The sequencing was performed on the Illumina iSeq platform as part of a flowcell (2 \times 151, 300 cycles; Illumina). Sequence trimming and *de novo* assembly was performed in CLC Genomics Workbench version 12.0.3. Blastn was used to search for sequence similarity against the nonredundant National Center for Biotechnology Information (NCBI) databases.¹¹

Gene annotation and sequence similarity

Open reading frames (ORFs) were identified automatically with GeneMark and Glimmer and revised manually in DNA Master.^{12–14} The genomes were searched for tRNA sequences using Aragorn and tRNAscan-SE.^{15,16} The ORFs were manually annotated using both HHPred against three downloaded databases (pFam 32.0, SCOP70 1.75 and PDB70) and blastp against the NCBI nonredundant databases.¹⁷ The nucleotide similarity heatmap between Bertil, Misse, and Strit and *Autographiviridae* phages was made with viridic (default settings).¹⁸ Gene synteny and nucleotide similarity were compared with Easyfig version 2.2.2. (default blastn settings with 2.9.0+ NCBI blast).¹⁹ Phylogenetic analyses of the predicted RNA polymerase, major capsid protein and large terminase were performed with MEGAX version 10.1.8.²⁰ The 20 most similar proteins from other phages, found by blastp, were included in the trees. The RNA polymerase, major capsid protein and large terminase proteins of the *Autographiviridae* phages included in Figure 2 were also included in the phylogenetic analyses. The proteins were aligned with MUSCLE, and the phylogenetic distances were inferred using a maximum likelihood method. The reliability of the trees was tested using bootstrap with 100 replicates. The

assembled and annotated genomes of Bertil, Misse, and Strit were uploaded to the NCBI with accession numbers MW286266, MW286267, and MW286268, respectively.

Transmission electron microscopy

To obtain pure phage lysates for electron microscope pictures, 10 μL of phage lysates containing 10⁸ plaque forming units (PFU)/mL were further propagated in 1 L lysogeny broth (LB) medium (10 mM of MgCl₂ and CaCl₂) with 100 μL host overnight culture and left on a shaking table at 200 rpm overnight. The concentrated lysates were centrifuged (5500 \times g, 10 min, 4°C). Fifty-eight grams NaCl followed by 100 g PEG 8000 were added directly to the supernatant. The resulting phage-PEG suspension was shaken overnight (200 rpm, 5°C). The PEG suspension was centrifuged again, and the pellet was resuspended in 5 mL SM-buffer and incubated overnight. The resuspended PEG pellet was centrifuged (10,000 \times g, 10 min, 4°C) and the pellet discarded. The phage particles were then purified in a cesium chloride gradient, as described elsewhere.²¹ For obtaining electron micrographs, 100 μL of the CsCl phage suspension were dialyzed against SM-buffer on a Millipore VSWP 0.025 μm filter membrane (Merck, Darmstadt, DE) for 30 min. The dialyzed phages were allowed to adsorb to ultrathin carbon films for 30 min and then fixed with 1% (v/v) glutaraldehyde (EM-grade) for an additional 30 min. Negative staining was performed with 2% (w/v) uranyl acetate. The ultrathin carbon films were picked up with standard 400-mesh copper grids (3 mm diameter). The transmission electron microscopy was performed using an accelerating voltage of 80 kV (Tecnai 10; FEI Thermo Fisher Scientific, Eindhoven, The Netherlands) and micrographs were taken with a MegaView G2 CCD camera (Emsis, Muenster, Germany).

Host-range screening and efficiency of plating

The phages were originally isolated with *P. syringae* GAW0113. The plaque forming ability of the three phages were additionally tested against all 13 strains of *P. syringae*: GAW0113, CC0663, CC1417, CC1513, CC1532, CC1557, CC1630, CC94, CFBP2341, CFBP4407, CLA0302, R4_A29-2, and UB246.²² The original isolation host, GAW0113, was included as a positive control and for comparison. The phages were propagated as stated earlier to a final titer of 10⁸–10⁹ PFU/mL. The phage lysates were used in a double agar overlay assay with 13 strains of *P. syringae*. The lysates were spotted (10 μL /spot) on LB plates with 100 μL overnight culture of the 13 different hosts and the isolation host and incubated overnight at room temperature. The relative efficiency of plating was measured for the sensitive strains and the original isolation host. The phage lysates were serially diluted 10-fold to 10⁻¹⁰ and a 5 μL aliquot of each dilution step was spotted on three separate LB plates for each host strain. Efficiency of plating (EOP) was calculated as the titer relative to that of the original isolation host.

Results

Genomics

The genomes of the three phages are all double-stranded DNA, with sizes of 41,373, 41,342, and 41,374 bp for Bertil, Misse, and Strit, respectively. The GC-content is 58% for

all three phages. All the genomes contain inverted terminal repeats of slightly varying length (307, 302, and 305 bp, respectively). The positions of the terminal repeats, and thus the genome start sites, were identified by the presence of two distinct spikes in read-start coverage at two nearby sites in the genome, encompassing areas with approximately double coverage of reads for that position compared with the rest of the genome. Bertil and Strit are almost identical except one deletion and nine single nucleotide mutations including a missense mutation (p.His97Gln) in the DNA polymerase gene. The other eight mutations and the deletion are either silent or in noncoding regions. Misse lacks a 195 bp ORF located in the middle of the genomes of Bertil and Strit, but contains an additional 100 bp noncoding region (Fig. 1). No function could be assigned for the ORF only found in Bertil and Strit. In total the phage genomes contain either 50 or 51 ORFs, all on the same strand and in the same reading direction. As many as 27 ORFs could be annotated with a putative function for each of the phages (Fig. 1). There was limited sequence similarity between the newly isolated phages and other phages of *Autographiviridae* on the nucleotide level (Fig. 2), but gene synteny and gene size seems to be conserved for most of the predicted core genes involved with replication, DNA packaging, and capsid-assembly and

lysis (Fig. 2). The similarity in gene synteny is, however, limited for many other putative ORFs with no predicted product (Fig. 1). No integrases or other genes related to a lysogenic phase were found in any of the three genomes. No tRNA sequences were found in any of the three genomes.

Virion morphology

Because of high genetic similarity between Bertil, Misse, and Strit, only one phage, Strit, was selected for transmission electron microscopy. Although the function of the missing gene in Misse is unknown, it is presumed that Misse and Bertil share the same or very similar capsid structure as Strit. Transmission electron microscopy (with seven phage particles) shows that Strit possesses an isometric head (diameter: 58 nm) and a short cone-shaped tail stub (length: 13 nm) (Fig. 3).

Nucleotide similarity to other phages

A nucleotide comparison using blastn, to available sequences in the NCBI NR database, shows that the three genomes have the largest similarity with *Sphaerotilus* phage yB_SnaP_R1 (21% query coverage and 67.02% identity) belonging to the *Autographiviridae* family. The blastn resulted in 126 out of 128

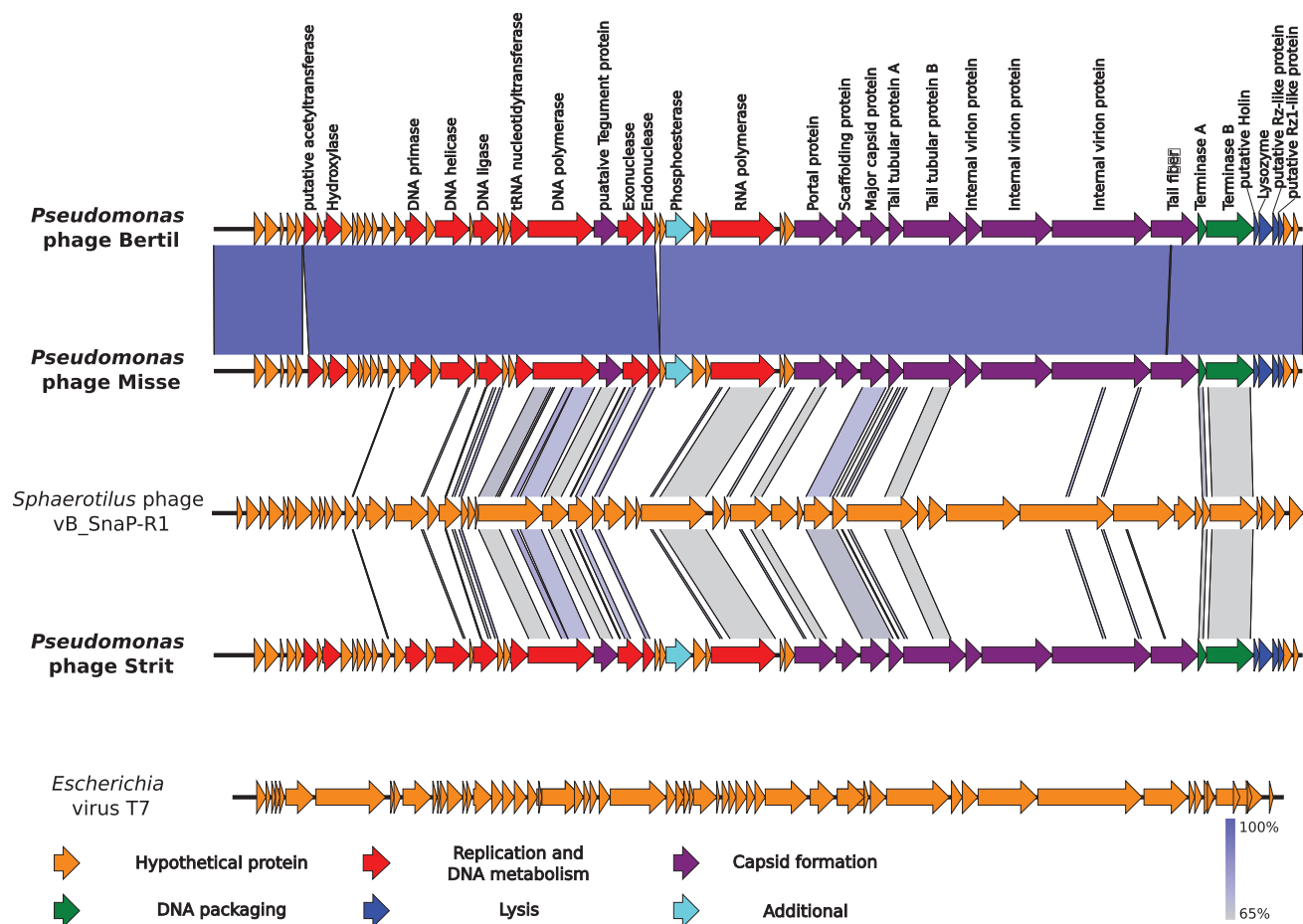


FIG. 1. Comparison between *Pseudomonas* phages Bertil, Misse, and Strit and two *Autographiviridae* phages with Easyfig software (default blast settings). *Sphaerotilus* phage yB_SnaP-R1 has the highest nucleotide similarity with Bertil, Misse, and Strit and *Escherichia* virus T7 is shown for comparison of gene synteny as one of the most studied *Autographiviridae* phages. Open reading frames are represented by arrows indicating the reading direction. Pairwise nucleotide similarity between the different genomes is shown by color (from gray to blue). The predicted gene products for Bertil, Misse, and Strit are shown above the genome of Bertil. Colors denote protein classes as described in the figure.

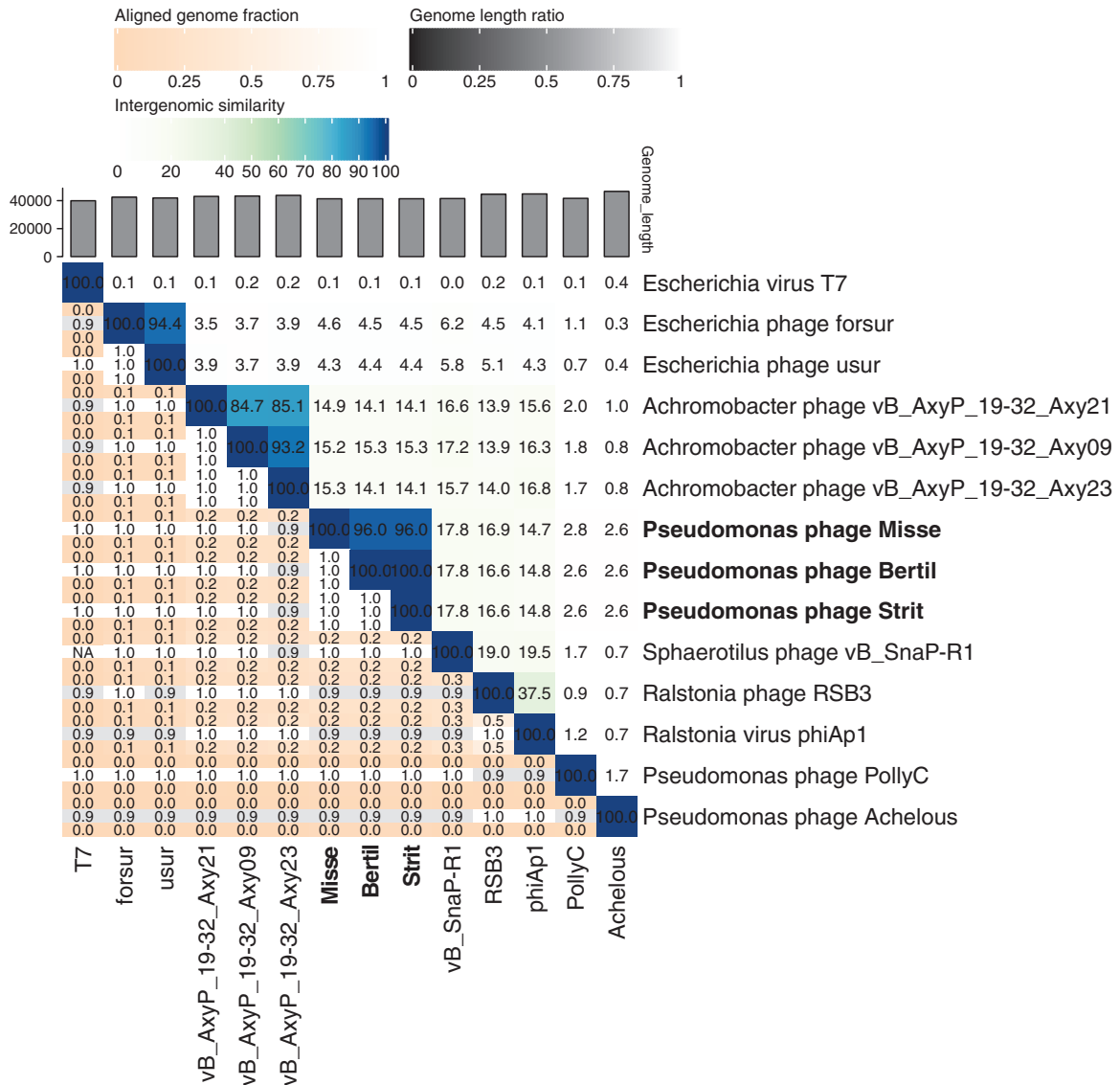


FIG. 2. Pair-wise nucleotide comparisons.

hits against phages in the Caudovirales order with 123 of the hits against *Autographiviridae* phages. Phylogenetic trees of the RNA polymerase, major capsid protein, and large terminase show that Bertil, Misse, and Strit also form their own cluster apart from other *Autographiviridae* phages.

Phage host range

The three phages were able to lyse and form plaques with similar efficiency against 3 out of 13 *P. syringae* strains including GAW0113 against which it was originally isolated

(Table 1). The variation in EOP between the different host strains were <10-fold for all three phages, despite a starting titer between 10⁸ and 10⁹ PFU/mL (Table 1). None of the phages had a significantly increased EOP for either of the tested strains compared with the isolation host (Wilcoxon Rank-Sum test, *p*-values >0.05).

Discussion

Phage genomes are difficult to compare on a nucleotide level because of their high diversity. Gene synteny and core

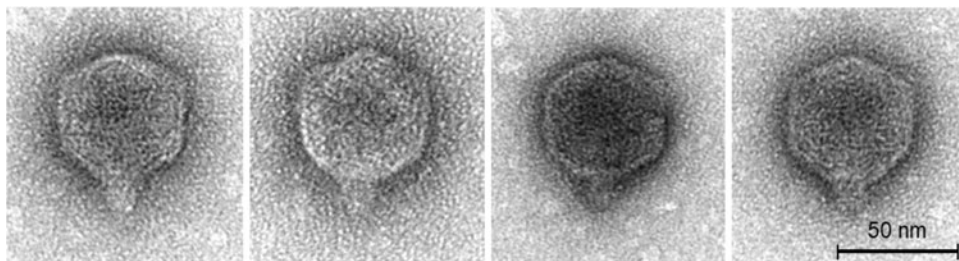


FIG. 3. Transmission electron micrographs.

TABLE 1. HOST RANGE ASSAY

Host strain	Phylogroup	Phage		
		Bertil	Misse	Strit
CC1630	01	—	—	—
CC94	02	—	—	—
R4_A29-2	03	—	—	—
CC1513	04	—	—	—
CFBP2341	05	—	—	—
CC0663	07	—	—	—
CC1532	09	5 ± 1.3	3.9 ± 0.6	1.6 ± 0.6
CC1417	09	2.3 ± 1.4	2.1 ± 0.2	1.6 ± 1.1
CC1557	10	—	—	—
CFBP4407	11	—	—	—
GAW0113	12	1 ± 0.2	1 ± 0.6	1 ± 1.0
UB246	13	—	—	—
CLA0302	13	—	—	—

proteins are, however, often conserved even in phages with low nucleotide similarity.²³ Most genes required for assembly of the procapsid, capsid, tail fibers, and DNA packaging have been predicted in Misse, Bertil, and Strit, but no proteins with homology to coat proteins have been found. Neighboring *holin* and *endolysin* genes were predicted in all three phages (gp49 and gp50, respectively). gp51 shows a weak similarity to a Rz-like protein from *Escherichia* virus T7 (highest *E*-value = 0.003) based on blastn. Interestingly, gp51 also shows a high similarity to several genes placed directly upstream of a gene encoding a lysozyme in other *Autographiviridae* phages. A small potential ORF was found overlapping both gp51 and gp52 in all three phage genomes. The product of these genes shows a similarity to putative Rz1-like proteins. Based on this, the phage genomes contain lysis cassettes similar to that of *Escherichia* virus T7 and found in many phages targeting gram-negative bacteria.²³

Phages have originally been classified based on the morphology of phage particles or host range, but the basis of phage taxonomy has gradually shifted to genetic analyses.²⁴ Based on this, the International Committee on Taxonomy of Viruses (ICTV) recently changed the taxonomy of the Caudovirales order, so that the *Autographiviridae* is now a family within the Caudovirales order. Bertil, Misse, and Strit have basic characteristics, such as a genome size of 41 kb with inverted terminal repeats, as well as morphological traits similar to the known characteristics of the *Autographiviridae* phages.²⁵ The three phages also present the highest nucleotide level similarity to phages within the *Autographiviridae* family. However, the low similarity to other phages makes it difficult to place the phages within an existing subfamily or genus. The three phages could, therefore, constitute a new genus within the *Autographiviridae* family. This is corroborated by phylogenetic analysis of the predicted RNA polymerase, major capsid protein, and large terminase (Supplementary Fig. S1). For all three proteins, Bertil, Misse, and Strit form their own cluster among other *Autographiviridae* phages. The separate branching of Bertil, Misse, and Strit is most reliable for the major capsid protein and the large terminase.

The *P. syringae* species complex has been classified in 13 phylogroups as well as additional subgroups based on the multilocus sequence typing of four housekeeping genes.²² Although not all strains have been tested for pathogenicity against crops,

many strains of *P. syringae* from nonagricultural origin are nevertheless potential pathogens.⁵ The original isolation strain, GAW0113, belongs to phylogroup 12, which comprises only a small number of strains. The two other sensitive strains tested in the host-range assay, CC1417 and CC1532, belong to phylogroup 9 (Table 1). Although none of the sensitive strains have been tested for pathogenicity on plants, CC1417 can induce a hypersensitive reaction on tobacco leaves unlike CC1532 and GAW0113.²⁶ Successful infection replication of phage is influenced by many factors such as host cell receptors and compatibility with the host replications machinery. Bacteria have also developed an array of different antiphage defense mechanisms, such as CRISPR-Cas, that influence the susceptibility of a bacteria toward phage infection.²⁷ Despite the genetic diversity of the hosts, the difference in EOP was <10-fold between the three sensitive *P. syringae*. Berge et al.²² have previously shown that the different phylogroups share many phenotypic traits despite being genetically distinct and this seems to include sensitivity toward specific phages.

All three sensitive strains have been isolated from aquatic habitats in either France or the United States.²² This indicates that the characteristics influencing phage susceptibility are shared by some *P. syringae* strains independent of phylogroups or geographic distribution. It has been suggested that bacterial metabolic membrane-proteins are often used by phages for adsorption and the environment of the host bacteria could, therefore, influence phage host range.²⁸ However, populations of *P. syringae* mix between different habitats, so this parameter alone does not explain the shared phage susceptibility and more detailed analyses are needed to elucidate the phage–host mechanisms.²⁹ The lack of genes associated with the lysogenic cycle and the ability to target multiple strains within the *P. syringae* species make Bertil, Misse, and Strit relevant as antimicrobial therapeutic agents. The low relatedness to other phages could be an advantage for using one of the three phages as part of a larger phage cocktail. However, as they are mostly identical it is probably redundant to apply more than one of the three phages together.

Conclusion

The three isolated phages are highly similar, with Misse being the most distinct on a nucleotide and protein level. They all share many genomic and morphological features with other phages of the newly established *Autographiviridae* family, but limited nucleotide similarity. Based on morphology, nucleotide similarity and homology of predicted core proteins, Bertil, Misse, and Strit seem to be three phage isolates within their own genus of distinct *Autographiviridae* phages. All three phages were able to infect and lyse multiple strains of *P. syringae* across different phylogroups.

Acknowledgment

Angela Back (Max Rubner-Institut) is thanked for her technical assistance with the electron microscopy.

Authors' Contributions

Conceptualization by J.B.J., A.M.D., A.B.C., and L.H.H.; methodology by J.B.J., A.M.D., A.B.C., W.K., H.N., and L.H.H.; validation by J.B.J., A.M.D., A.B.C., W.K., and L.H.H.; formal analysis by J.B.J., H.N., and W.K.; investigation

by J.B.J., A.M.D., and A.B.C.; resources by W.K. and L.H.H.; data curation by J.B.J., A.M.D., and A.B.C.; writing—original draft preparation by J.B.J.; writing—review and editing by J.B.J., A.M.D., A.B.C., W.K., H.N., C.M., and L.H.H.; visualization by J.S.P. and H.N.; supervision by A.M.D., A.B.C., W.K., and L.H.H.; project administration by W.K. and L.H.H.; funding acquisition by W.K. and L.H.H. All coauthors have reviewed and approved of the article before submission.

Author Disclosure Statement

No competing financial interests exist.

Funding Information

This study was part of the “Phytoprotect” project funded by the Danish Research Council for Technology and Production Grants (Grant No. DFF-4184-00070), the Human Frontier Science Program (Grant No. RGP0024/2018), and the Villum Experiment (Grant No. 17595).

Supplementary Material

Supplementary Figure S1

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