American Journal of Potato Research (2020) 97:447–449
https://doi.org/10.1007/s12230-020-09793-1

SHORT COMMUNICATION

Complete Genome Sequence of *Ralstonia* Phage Remenis, a Member of Putative New Genus within the *Siphoviridae*

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Received: 26 February 2020 / Revised: 26 June 2020 / Accepted: 20 July 2020 / Published online: 24 July 2020

Abstract

One of the most destructive diseases affecting potato production globally is bacterial wilt, caused by *Ralstonia solanacearum*. Options for controlling this pathogen are currently limited. Therefore, in this study, whole genome sequence was used to assess the potential of phage Reminis as biocontrol agent. No sequences related to undesirable genes including virulence factors, antibiotic resistance, or lysogenic mediated or toxin-coding genes were found in phage genome, this suggests that phage Reminis may be a potential candidate for future *R. solanacearum* control strategies.

Resumen

Una de las enfermedades mas destructivas que afectan la producción de papa globalmente es la marchitez bacteriana, causada por *Ralstonia solanacearum*. Las opciones para controlar a este patógeno son actualmente limitadas. Por ello, en este estudio, se usó la secuencia genómica para analizar el potencial del fago *Reminis* como agente de biocontrol. En el genoma del fago no se encontraron secuencias relacionadas con genes "indeseables" que incluyeran factores de virulencia, resistencia a antibiótico, genes de lisogenia o genes que codifiquen toxinas. Esto sugiere que el fago *Reminis* pudiera ser un candidato potencial para futuras estrategias de control de *R. solanacearum*.

Keywords Potato brown rot · Biocontrol potential · Bacteriophage, genetic safety assessment

Phytopathogenic bacteria are diverse and cause many types of the most economically significant plant diseases worldwide. *Ralstonia solanacearum* is one of the most destructive and widespread plant pathogenic bacterium worldwide. This pathogen is the causal agent of bacterial wilt (also known as brown rot on potato), one of the most devastating diseases of potatoes because it induces rapid and fatal symptoms (Messiha et al. 2019).

Management of *R. solanacearum* is difficult using conventional methods hence incorporation of bacteriophages in an integrated programme would be a promising approach (Wei et al. 2017). However, not all phages are considered suitable for biocontrol applications. A suitable phage for biocontrol must be strictly lytic, non-transducing, and undesirable genes should be absent (Fernández et al. 2019). Here, we report the isolation and genome sequence of bacteriophage Reminis, a novel siphovirus capable of infecting *R. solanacearum* strains.

This phage was isolated from irrigation water in a potato field by the double agar overlay procedure. Negative staining transmission electron microscopy (TEM) was used to visualize the morphology of virion. Genomic DNA was isolated from phage lysates using SDS/proteinase K extraction method (Green and Sambrook 2018) and then sequenced on the Illumina NextSeq 500 platform (Illumina, USA) with an average read length of 2 × 150. Phage termini were predicted from raw sequencing reads using PhageTerm version 1.0.8 (Garneau et al. 2017). Low-quality bases and adapters were removed using Trimmomatic v0.38 (Bolger et al. 2014) based

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Electronic supplementary material The online version of this article (https://doi.org/10.1007/s12230-020-09793-1) contains supplementary material, which is available to authorized users.

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on quality cutoff Phred score of ≥ 30. *De novo* genome assembly was performed using CLC Genomics Workbench (CLCbio, QIAGEN, version 20.0). We performed assembly validation in CLC Genomics using clc_mapper with default settings, considered a percentage > 95% of reads mapping to the genome, and average whole genome coverage least 100×.

The prediction of open reading frames (ORFs) was performed using GeneMark server with a length threshold of 90 bp, and the putative functions of the ORFs were determined by BLASTp against the nonredundant NCBI database and InterProScan searches. The presence of tRNA was scanned through tRNAscan-SE search server. Additionally, all identified ORFs were compared against the Virulence Factor Database (VFDB) (Chen et al. 2015) and Comprehensive Antibiotic Resistance Database (CARD) (Jia et al. 2016). BLASTN was used to find genomes related to Reminis. Pairwise phage genomes comparison was done with ClustalW. We performed phylogenetic analysis based on the deduced amino acid sequence of the terminase large subunit and DNA polymerase of the Reminis compared with best BLASTp (E-value cutoff of 1×10^{-5}) hits to homolog proteins from other phages. We constructed multiple sequence alignments using MUSCLE with the default parameters. Phylogenetic tree was inferred using the neighbor-joining method with 1000 bootstrap re-samplings in Geneious Prime software.

TEM revealed that phage belonged to family *Siphoviridae* (icosahedral head with long, flexible, non-contractile tail). PhageTerm suggested that the 5' and 3' ends of the Reminis genome were characterized by two short direct terminal repeats (DTRs) of 377 bp. Because DTRs that flank virus genome termini are characteristic of linear double stranded bacteriophage genomes (Casjens and Gilcrease 2009), the Reminis genome therefore consiste of a linear dsDNA of 45,228 bp with a GC content of 42.7%, 63 predicted ORFs, putative function could be assigned to 30 of them, including proteins responsible for the viral morphogenesis, DNA packaging, replication, and host cell lysis, no tRNAs-encoding sequences were found. Moreover, bioinformatics analyses revealed that phage genome did not encode undesirable genes, such as antibiotic resistance, lysogeny, transducible elements, toxins or other virulence factors, which suggests that Reminis is a potentially safe biological agent to improve the biocontrol efficacy of *R. solanacearum*.

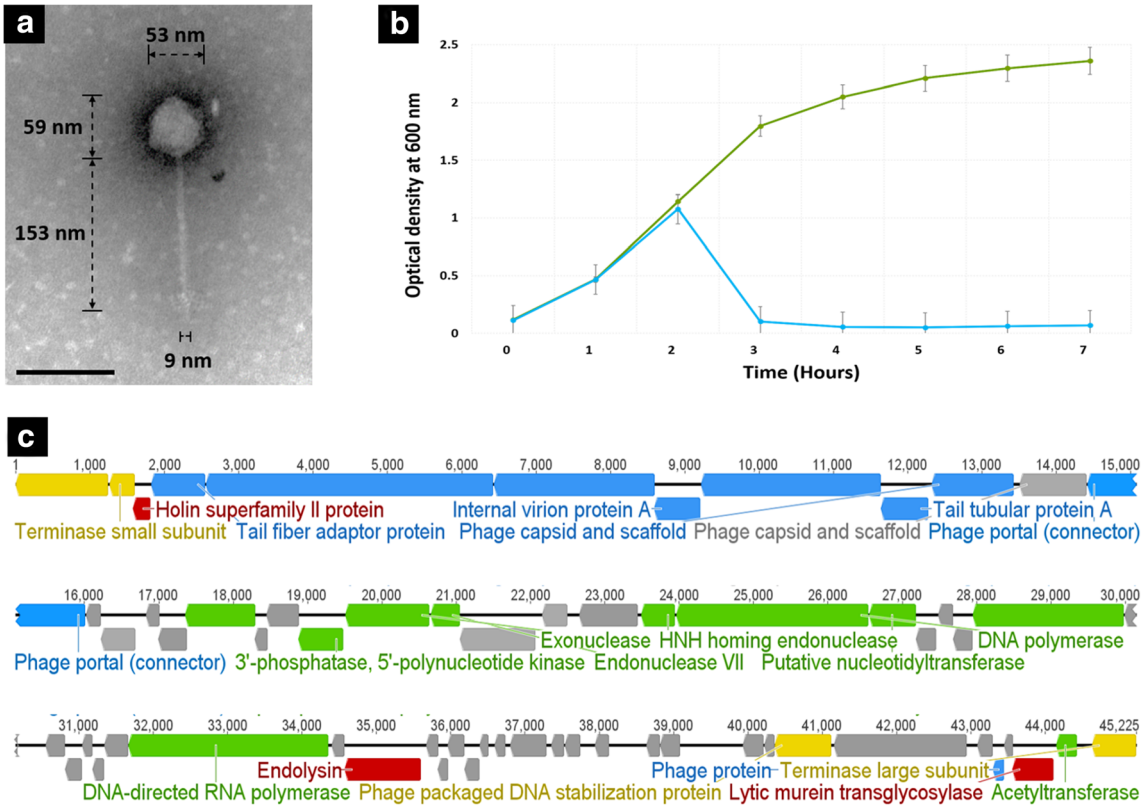

Fig. 1 **a** Morphology of phage Reminis. The phage was stained with 2% phosphotungstic acid and visualized at 100,000 × magnification with transmission electron microscopy. Scale bars represent 100 nm. **b** *In vitro* bacterial lytic activities of phage Reminis at a MOI of 0.001. For the time-kill experiments logarithmic-phase growing *R. solanacearum* cells were exposed Reminis in TSB medium for 7 h at 28°C (blue line). As a control, TSB was inoculated with *R. solanacearum* (green line). The optical density at 600 nm was determined at each timepoint. Each point represents the means ± SD of three replicate experiments. **c** Functional genome map of phage Reminis. The arrows indicated open reading frame (ORF), the orientation of which shows the direction of transcription. The color of each ORF refers to the functional category: phage structure (blue), DNA regulation module (green arrows), packaging module (yellow), host lysis proteins (red arrows), and hypothetical proteins (gray arrows)

Genome sequencing revealed that the phage Reminis displays high nucleotide sequence divergence to other phages genomes in public databases, showed that the best hits included *Escherichia* phage ECBP5 (a member of the *Podoviridae* family) with only 5% query coverage and 75% identity. At the phylogenetic level, it is not unusual that phages of different morphology, belonging to different families, to be closely related phylogenetically. Brussow and Desiere (2001) revealed that the genome of *Siphoviridae* from the λ supergroup are more closely related to P22-like *Podoviridae*. In fact, some researchers suggest that the validity of the family *Podoviridae* may be questioned because the main difference between the two families, tail length, may be due to the presence or absence of a tail length ruler gene (Kutter and Sulakvelidze 2005).

Phylogenetic analysis based on terminase large subunit and DNA polymerase showed that Reminis clusters independently from the other studied phages (Supplementary material). This finding suggests that Reminis is significantly different from other reported phages, could support the proposal of a new genus.

In order to an accurate prediction of phage Reminis taxonomic classification, pairwise genome alignment was performed between both phages using ClustalW. Genome analysis revealed that these phages shares only 42.2% identity. According to the International Committee on Taxonomy of Viruses (ICTV), a bacteriophage genus is defined as a group of phages that share at minimum 50% DNA identity (Adriaenssens and Brister 2017). Based on the above, we suggest the creation of a new genus within the *Siphoviridae* family, and Reminis should be considered as a founding member.

The complete genome sequence of *Ralstonia* phage Remenis was deposited in GenBank under the accession number MN478376. The raw sequence reads are available in the SRA database under accession number SRR11712017 (BioProject number PRJNA630568) (Fig. 1).

Acknowledgements Support for the research was provided by the CONACYT (Project no. 251516). The autors appreciates the excellent technical assistance of Ing. Oscar Patrón and Luis Figueroa.

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