First Isolation and Molecular Characterization of Bacteriophages Infecting *Acidovorax citrulli*, the Causal Agent of Bacterial Fruit Blotch

Aryan Rahimi-Midani¹, Yong Seok Lee², Se-Won Kang³, Mi-Kyeong Kim⁴, and Tae-Jin Choi^{1*}

¹Department of Microbiology, Pukyong National University, Busan 48513, Korea

²Department of Life Science and Biotechnology, Soonchunhyang University, Asan 31538, Korea

³Biological Resources Center, Korea Research Institute of Bioscience and Biotechnology, Jeongseup 56212, Korea

⁴Department of Clinical Laboratory Science, Gimhae College, Gimhae 50811, Korea

(Received on August 29, 2017; Revised on October 26, 2017; Accepted on November 2, 2017)

Bacteriophages of *Acidovorax citrulli*, the causal agent of bacterial fruit blotch, were isolated from 39 watermelon, pumpkin, and cucumber leaf samples collected from various regions of Korea and tested against 18 *A. citrulli* strains. Among the six phages isolated, ACP17 forms the largest plaque, and exhibits the morphology of phages in the *Myoviridae* family with a head diameter of 100 ± 5 nm and tail length of 150 ± 5 nm. ACP17 has eclipse and latent periods of 25 ± 5 min and 50 ± 5 min, respectively, and a burst size of 120. The genome of ACP17 is 156,281 base pairs with a G + C content of 58.7%, 263 open reading frames, and 4 transfer RNA genes. Blast search and phylogenetic analysis of the major capsid protein showed that ACP17 has limited homology to two *Stentrophomonas* phages, suggesting that ACP17 is a new type of *Myoviridae* isolated from *A. citrulli*.

Keywords : BFB, phage, watermelon

Handling Associate Editor : Oh, Chang-Sik

Bacterial fruit blotch (BFB) is a bacterial disease of many *Cucurbitaceae* family members caused by *Acidovorax citrulli* (Schaad et al., 2008) [formerly *Acidovorax avenae*

subsp. *citrulli* (Willems et al., 1992)]. After large economic losses in southwestern Indiana, USA, in the 1980s (Latin and Fane, 1990), this disease became a major economic threat to watermelon, melon, and cucumber crops in South America, Australia, and Asia (Isakeit et al., 1997; Schaad et al., 2008). In Korea, BFB was first reported in 1991 on watermelon in Gochang, Jeollabuk-do province (Song et al., 1991). Today, occasional outbreaks cause economic problems, and several lawsuits are ongoing between growers and seed companies.

BFB is known to be a seed-borne disease, and seed infestations are the result of blossom contamination with *A. citrulli*, which can localize in the embryo and endosperm tissue of seeds (Lessl et al., 2007). This bacterium causes seedling blight, necrosis of the true leaves, extensive stem necrosis, and fruit blotch. On fruit, water-soaked lesions progress through the rind, and the fruit decays and cracks. Sometimes this happens close to harvest time, causing devastating and unrecoverable losses.

Chemical treatments, such as copper-containing bactericide/fungicide application on infested fields, are ineffective (Hopkins, 1991), and there is currently no complete and monogenic resistant cultivar available. Because the disease is seed-borne, use of pathogen-free seed is a priority for disease control. Seed can be produced in areas with cool and dry climates that have a lower chance of BFB development. Seed can be treated with antibiotics and disinfectants such as streptomycin, sodium hypochlorite, and hydrochloric acid (Hopkins et al., 1996, 2003). However, as *A. citrulli* is localized under the seed coat (Lane and Latin, 1992), the control efficacy of external treatments varies widely (Hopkins et al., 2003; Rane and Latin, 1992). Treatment of the seed at 85° for 3-5 days can effectively remove the pathogen, but can reduce germination (Kubota et al.,

*Corresponding author.

Phone) +82-51-620-6367, FAX) +82-51-611-6358

E-mail) choitj@pknu.ac.kr

© This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

2012).

Recently, ‘phage therapy’ or ‘bacteriophage biocontrol’ of plant disease, which are applications of bacteriophages for control of bacterial disease, is attracting public attention due to the prevalence of drug resistant bacteria and contamination of food and the environment with over-used antibiotics (Sharma et al., 2017). In this study, we isolated and characterized bacteriophages that infect *A. citrulli* for the first time, which can be used bacteriophage biocontrol of BFB.

Eighteen strains of *A. citrulli* were kindly provided by Professor Changsik Oh of Kyung Hee University and were genotyped using a PCR assay with the primers G2AcFwd (5′-CGATAGGGTTGGGTTCAAG-3′), G12AcFwd (5′-CCGAAGAGATAACACTGCATC-3′) and G12AcRev (5′-ACGTAAGCGGATTTTTC-3′) (Zivanovic and Walcott, 2016). *A. citrulli* strains were grown on King’s medium B Base (KB) at 34°C for 48 h.

Samples for phage isolation were collected from 96 sites around the Korean Peninsula. Samples included soil from watermelon farms, and leaves of watermelon, pumpkin, and cucumber plants. Approximately 10 g of leaf or soil sample was mixed with sterilized distilled water for 30 min, then centrifuged at $3000 \times g$ for 20 min at 4°C. The supernatant was filtered through Minisart filters (Sigma-Aldrich, St. Louis, MO, USA) with a 0.2- μ m pore size. The presence of phages was determined through a spotting assay against different *A. citrulli* strains. For the spotting assay, 300 μ l of exponential phase *A. citrulli* was mixed with 4 ml KB top agar, overlaid on KB media, and left to solidify at room temperature for 20 to 30 min. Filtered sample water (3 μ l) was spotted on the solidified top agar and incubated at 34°C overnight. Among the 96 samples, six, including three watermelon leaves, two pumpkin leaves, and one cucumber leaf sample, were positive in the spot assay. The plaque assay was conducted three times using the wash solution described above to obtain a pure culture of bacteriophages. Plaques were picked using sterilized pipette tips with the ends cut, and were maintained in SM buffer (100 mM NaCl, 8 mM MgSO₄, 50 mM Tris-Cl). Considering the large number of samples, the percentage corresponding to six positive samples (6.25%) is quite low. Although it has been reported that there are an average of 1.5×10^7 phages per gram of soil (Ashelford et al., 2003), it is estimated that there are up to over 10^9 bacterial cells in the same amount of soil (Torsvik and Øvreås, 2002). Thus, the chance of isolating phages that infect a specific bacterium is still low. One method to overcome this problem is enrichment of the phage population in samples through addition of the proper medium for the bacterial host, along with

the corresponding host or a mixture of host strains, followed by incubation and phage isolation by plaque assay. In the following experiment, we performed these enrichment steps and were able to isolate several phages. On the other hand, isolation of all six phages from leaves was an unexpected result, considering the harsh conditions, such as UV radiation, present on the leaf surface. The phyllosphere is known to be the location where phages can most readily be isolated, although how the phages get there is not yet understood. It has been suggested that they originate in the soil from which the plant germinated or are deposited by insect vectors (Buttimer et al., 2017).

It is known that bacteriophages that produce larger plaques have bigger burst sizes (Abedon and Culle, 2007). If a phage is going to be used for bacteriophage biocontrol, it will be necessary to produce a large amount of phages. Therefore, we selected a phage called ACP17, which produced the largest plaques among the six phages isolated. The one-step growth curve also revealed that the eclipse and latent periods of ACP17 were 25 ± 5 min and 50 ± 5 min, respectively and the burst size was 120.

Phage ACP17 was amplified for EM observation and genome analysis following previously published protocols with minor modifications (Bonilla et al., 2016), including ultracentrifugation on a CsCl density gradient of 1.25, 1.37, and 1.50 g/ml. Purified phage ACP17 was placed on 300 mesh copper grid, negatively stained with 4% uracil acetate, and observed with a transmission electron microscope (H-7500, Hitachi, Ltd., Tokyo, Japan) at the Center for Research Facilities of Pukyong National University. Phage sizes were measured using the software ImageJ (<https://imagej.nih.gov/ij/>). EM observation showed a clear picture of a head measuring 100 ± 5 nm and tail 150 ± 5

Fig. 1. Electron micrograph of ACP17 using negative staining.

nm in length, indicating that phage ACP17 is a member of the family *Myoviridae* (Fig. 1). The family *Myoviridae* is composed of diverse phages with different burst sizes and infection processes. Thus, the burst size, eclipse period, and latent period are within the ranges of viruses in the *Myoviridae* family. For example, bacteriophage J2, which infects the plant pathogen *Ralstonia solanacearum*, belongs to the *Myoviridae* family and has a latent period of 60 min and burst size of 100-110 plaque-forming units (PFU) per infected cell showed a similar one-step growth curve to ACP17 (Bhunchoth et al., 2015).

One possible application of ACP17 is for control of BFB. There are two genotypes of *A. citrulli*, genotype I and II (Tao et al., 2009), which can be differentiated via PCR, as described above. ACP17 was tested for its lytic activity against 18 host strains; 16 of genotype I and 2 of genotype II. As shown in Table 1, ACP17 could lyse all 16 strains of genotype I and one strain of genotype II. Between the two genotypes, genotype II is the major causal agent of BFB in watermelon. Although the number of tested genotype II strains is limited, the inability of ACP17 to lyse all genotype II strains poses a problem for the application of this phage in bacteriophage biocontrol. However, more extensive investigation using more host strains, especially of genotype II, is necessary.

One concern for the use of bacteriophage biocontrol in plant disease is the stability of phage particles. Although

purified phage can be applied directly, it can also be mixed with other chemical components, such as antibiotics for their synergistic action (Borah et al., 2000; Frampton et al., 2012). After application, phages will face harsh conditions and the stability of phages is important for therapeutic application. Among various external physical and chemical factors that affect the survivability of bacteriophages, temperature and pH are crucial (Jończyk et al., 2011). The stability of ACP17 was tested at different pH and temperature levels. Purified ACP17 was added (approximately 4.0×10^5 PFU/ml) to SM buffer that had been adjusted to pH 2, 5, 6, 7, 9, or 12 and incubated for 24 h at room temperature prior to plaque assays with the *A. citrulli* strain NWB SC196. As shown in Fig. 2, ACP17 was stable over the pH range of 6 to 9, but the titer decreased at pH 5 and showed complete inactivation at pH 2 and 12. Similarly, the stability of ACP17 was tested at different temperatures by incubating the phage (approximately 4.0×10^5 PFU/ml) for 24 h at -20 , 4, 25, 34, 37, 45, and 60°C before performing plaque assays. The results shown in Fig. 2 reveal that ACP17 is stable at temperatures ranging from -20 to 45°C , but is inactivated at 60°C . These results suggest that ACP17 will be stable during regular mixing processes, but heating at high temperature for formulation or long-term storage at high temperature should be avoided (Gill and Hyman, 2010;

Table 1. Host range of phage ACP17

Group	No	Strains	Host	Lysis activity of ACP17
I	1	NWB SC107	Watermelon	+
	2	NWB SC108	Watermelon	+
	3	NWB SC109	Watermelon	+
	4	NWB SC111	Watermelon	+
	5	NWB SC172	Watermelon, fruit	+
	6	NWB SC187	Watermelon	+
	7	NWB SC196	Watermelon, stem	+
	8	NWB SC201	Watermelon	+
	9	NWB SC206	Watermelon, stem	+
	10	NWB SC212	cucumber,	+
	11	NWB SC238	Watermelon, leaf	+
	12	NWB SC058	Melon	+
	13	NWB SC076	Watermelon	+
	14	NWB SC175	Cucumber	+
	15	NWB SC195	Watermelon, leaf	+
	16	NWB SC202	Watermelon	+
II	17	NWB SC074	Watermelon	–
	18	KHU	Watermelon	+

Fig. 2. Stability of ACP17 under various pH (top) and temperature (bottom) conditions analyzed by plaque assay. Results are the average of three replicates and the vertical bars indicate standard deviation.

Lim et al., 2013).

Total nucleic acid was isolated from the purified virus and subjected to digestion with DNase, RNase, and exonuclease. As expected, nucleic acid extracted from ACP17 was digested by DNase and exonuclease but not by RNase, indicating that the nucleic acid is linear double-stranded DNA (data not shown). Genomic library construction and sequence analysis of ACP17 was performed by Teragene Korea Co., Seoul, Korea. Purified genomic DNA was randomly sheared to yield DNA fragments with an average size of 350 base pairs (bp) using a Covaris S2 Ultrasonicator (Covaris, Inc., Woburn, MA, USA). Library preparation was performed using the Illumina TruSeq DNA PCR-free preparation kit (Illumina, Inc., San Diego, CA, USA) and sequenced on the Illumina HiSeq 4000 platform (Illumina, Inc., San Diego). Further image analysis and base calling

were performed with real time analysis (RTA) 2.7.3 and bcl2fastq v2.17.1.14 (Illumina, Inc., San Diego). Normalized reads were assembled using IDBA-UD ver. 1.1.1. (Peng et al., 2012) and contigs were aligned to the NCBI nucleotide database using BLASTN with an E-value cutoff of $1e-10$. Alignment results were classified taxonomically using Krona tools (Ondov et al., 2011). Bacteriophage contigs were extracted using an unshared program that we had developed. Gene models were predicted using GeneMark.hmm with Heuristic models (Besemer and Borodovsky, 1999).

Sequence analysis showed that the genome of ACP17, which is deposited in GenBank under accession number KY979132, is composed of 156,281 bp with G + C content of 58.7%, 263 open reading frames (ORF), and 4 transfer RNAs (tRNAs) (Supplementary Table 1). It also revealed

Fig. 3. Phylogenetic tree based on the amino acid sequences of the major capsid proteins of selected members of *Myoviridae* and phage ACP17. Bar indicates the number of nucleotide substitutions per site.

Fig. 4. Comparative analysis of phage open reading frames (ORFs) encoded by phage ACP17 and *Stenotrophomonas* phage IME-SM1 using the Easyfig 9 program (<http://mjsull.github.io/Easyfig/>). Arrows indicate the ORFs and their orientation and lines indicate ORFs with amino acid sequence homology.

that the average length of phage genes is 578.09 bp, with a minimum length of 123 bp and a maximum gene length of 5712 bp. Among the 263 ORFs, 250 are encoded by the plus strand and 13 by the minus strand, while 101 were predicted to encode proteins of known function such as viral structural proteins, proteins involved in virus replication, recombination, transcription, DNA modification, and virus release. In BLAST analysis, among the 101 putative proteins, only 37 and 4 showed homology to those encoded by *Stenotrophomonas* phage IME-SM1 and *Stenotrophomonas* SMP1, respectively. The high sequence homology to these two viruses was confirmed using a phylogenetic tree constructed based on the amino acid sequences of the major capsid protein. As shown in Fig. 3, ACP17 showed close relationships with these two phages.

There is no detailed information available about *Stenotrophomonas* phage IME-SM1, which is a member of the *T4virus* genus of family *Myoviridae*, and whose genome sequence was deposited under accession number KR560069.1. *Stenotrophomonas* phage SMP14 was isolated from *Stenotrophomonas maltophilia*, an opportunistic bacterium causing nosocomial infections. *Stenotrophomonas* phage SMP14 also belongs to the *Myoviridae* family; it has a head size of 87×77 nm, a tail of 129×18 nm, and a genome size of 160 kilobases (kb), which makes it smaller than ACP17 but with a similar genome size. Both *Acidovorax* and *Stenotrophomonas* belong to the phylum *Proteobacteria*, but fall into the different classes of *Gammaproteobacteria* and *Betaproteobacteria*, respectively. Therefore, the close relationship of ACP17 to these two phages might simply indicate that ACP17 is more closely related to this virus than other known viruses, and this relationship can be resolved after more viruses similar to

ACP17 have been isolated. In fact, alignment of the ORFs encoded by ACP17 and *Stenotrophomonas* phage IME-SM1 showed that ORFs common to both phages are a very limited component of the ORFs encoded by these two phages (Fig. 4). Therefore, we concluded that phage ACP17 is a novel phage first isolated from *A. citrulli*, and that it has limited homology to phages infecting *Stenotrophomonas*.

Despite homology to the structural proteins of *Stenotrophomonas* phages, the tail fiber protein of ACP17 showed the highest sequence homology to the *Vibrio* phage vB_VmeM-32. The ORF that encodes single-stranded DNA-binding proteins shares the highest amino acid sequence homology with that of *Prochlorococcus* phage P-SSM5. In addition, the putative RNA ligase encoded by ACP17 showed high amino acid sequence homology with that of *Ralstonia* phage RSP15 and *Salmonella* phage SSE121. In addition, ACP17 endolysin, which can digest the bacterial cell wall (Walmagh et al., 2013), exhibits homology to *Acinetobacter* phage vB_AbaS_TRS, isolated from *Acinetobacter baumannii* (Turner et al., 2016). Therefore, considering the limited homology of proteins encoded by ACP17 to those encoded by two *Stenotrophomonas* phages and the small number of proteins showing homology to those of other phages, ACP17 is assumed to be a new type of phage isolated for the first time from *A. citrulli*. The application of ACP17 in bacteriophage biocontrol against BFB will be considered in future research.

Acknowledgments

This work was supported by a Research Grant from Pukyong National University (year 2016).

References

- Abedon, S. T. and Culler, R. R. 2007. Optimizing bacteriophage plaque fecundity. *J. Theor. Biol.* 249:582-592.
- Ashelford, K. E., Day, M. J. and Fry, J. C. 2003. Elevated abundance of bacteriophage infecting bacteria in soil. *Appl. Environ. Microbiol.* 69:285-289.
- Besemer, J. and Borodovsky, M. 1999. Heuristic approach to deriving models for gene finding. *Nucleic Acids Res.* 27:3911-3920.
- Bhunchoth, A., Phironrit, N., Leksomboon, C., Chatchawankanphanich, O., Kotera, S., Narulita, E., Kawasaki, T., Fujie, M. and Yamada, T. 2015. Isolation of *Ralstonia solanacearum*-infecting bacteriophages from tomato fields in Chiang Mai, Thailand, and their experimental use as biocontrol agents. *J. Appl. Microbiol.* 118:1023-1033.
- Bonilla, N., Rojas, M. I., Cruz, G. N. F., Hung, S. H., Rohwer, F. and Barr, J. J. 2016. Phage on tap-a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. *PeerJ* 4:e2261.
- Borah, P. K., Jindal, J. K. and Verma, J. P. 2000. Integrated management of bacterial leaf spot of mungbean with bacteriophages of Xav and chemicals. *J. Mycol. Plant Pathol.* 30:19-21.
- Buttimer, C., McAuliffe, O., Ross, R. P., Hill, C., O'Mahony, J. and Coffey, A. 2017. Bacteriophages and bacterial plant diseases. *Front. Microbiol.* 8:34.
- Frampton, R. A., Pitman, A. R. and Fineran, P. C. 2012. Advances in bacteriophage-mediated control of plant pathogens. *Int. J. Microbiol.* 2012:326452.
- Gill, J. J. and Hyman, P. 2010. Phage choice, isolation, and preparation for phage therapy. *Curr. Pharm. Biotechnol.* 11:2-14.
- Hopkins, D. L. 1991. Chemical control of bacterial fruit blotch of watermelon. *Proc. Fla. State Hort. Soc.* 104:270-272.
- Hopkins, D. L., Cucuzza, J. D. and Watterson, J. C. 1996. Wet seed treatments for the control of bacterial fruit blotch of watermelon. *Plant Dis.* 80:529-532.
- Hopkins, D. L., Lovic, B., Hilgren, J. and Thompson, C. M. 2003. Wet seed treatment with peroxyacetic acid for the control of bacterial fruit blotch and other seedborne diseases of watermelon. *Plant Dis.* 87:1495-1499.
- Isakeit, T., Black, M. C., Barnes, L. W. and Jones, J. B. 1997. First report of infection of honeydew with *Acidovorax avenae* subsp. *citrulli*. *Plant Dis.* 81:694-694.
- Jończyk, E., Kłak, M., Międzybrodzki, R. and Górski, A. 2011. The influence of external factors on bacteriophages. *Folia Microbiol. (Praha)* 56:191-200.
- Kubota, M., Hagiwara, N. and Shirakawa, T. 2012. Disinfection of seeds of cucurbit crops infested with *Acidovorax citrulli* with dry heat treatment. *J. Phytopathol.* 160:364-368.
- Latin, R. X. and Rane, K. K. 1990. Bacterial fruit blotch of watermelon in Indiana. *Plant Dis.* 74:331-335.
- Lessl, J. T., Fessehaie, A. and Walcott, R. R. 2007. Colonization of female watermelon blossoms by *Acidovorax avenae* ssp. *citrulli* and the relationship between blossom inoculum dosage and seed infestation. *J. Phytopathol.* 155:114-121.
- Lim, J. A., Jee, S., Lee, D. H., Roh, E., Jung, K., Oh, C. and Heu, S. 2013. Biocontrol of *Pectobacterium carotovorum* subsp. *carotovorum* using bacteriophage PP1. *J. Microbiol. Biotechnol.* 23:1147-1153.
- Peng, Y., Leung, H. C., Yiu, S. M. and Chin, F. Y. 2012. IDBA-UD: a de novo assembler for single-cell and metagenomic sequencing data with highly uneven depth. *Bioinformatics*, 28:1420-1428.
- On dov, B. D., Bergman, N. H. and Phillippy, A. M. 2011. Interactive metagenomic visualization in a Web browser. *BMC Bioinformatics*, 12:385.
- Rane, K. K. and Latin, R. X. 1992. Bacterial fruit blotch of watermelon: association of the pathogen with seed. *Plant Dis.* 76:509-512.
- Schaad, N. W., Postnikova, E., Sechler, A., Clafflin, L. E., Vidaver, A. K., Jones, J. B., Agarkova, I., Ignatov, A., Dickstein, E. and Ramundo, B. A. 2008. Reclassification of subspecies of *Acidovorax avenae* as *A. Avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad et al., 1978) comb. nov., and proposal of *A. oryzae* sp. nov. *Syst. Appl. Microbiol.* 31:434-446.
- Sharma, S., Chatterjee, S., Datta, S., Prasad, R., Dubey, D., Prasad, R. K. and Vairale, M. G. 2017. Bacteriophages and its applications: an overview. *Folia. Microbiol.* 62:17-55.
- Song, W. Y., Kim, H. M., Son, I. Y. and Kang, Y. K. 1991. *Pseudomonas pseudoalcaligenes* subsp. *citrulli*: The causal agent of bacterial fruit blotch rot on watermelon. *Korean J. Plant Pathol.* 7:177-18.
- Tao, C., Guo-Liang, Q., Xiao-Li, Y., Jun-Yi, M., Bai-Shi, H. and Feng-Quan, L. 2009. Detection of a quorum sensing signal molecule of *Acidovorax avenae* subsp. *citrulli* and its regulation of pathogenicity. *Chin. J. Agric. Biotechnol.* 6:49-53.
- Torsvik, V. and Øvreås, L. 2002. Microbial diversity and function in soil: from genes to ecosystems. *Curr. Opin. Microbiol.* 5:240-245.
- Turner, D., Wand, M. E., Sutton, J. M., Centron, D., Kropinski, A. M. and Reynolds, D. M. 2016. A viable prophage isolated from *Acinetobacter baumannii* strain A118. *Genome announcement*. 4:e01051-16.
- Walmagh, M., Boczkowska, B., Grymonprez, B., Briers, Y., Drulis-Kawa, Z. and Lavigne, R. 2013. Characterization of five novel endolysins from Gram-negative infecting bacteriophages. *Appl. Microbiol. Biotechnol.* 97:4369-4375.
- Willems, A., Goor, M., Thielemans, S., Gillis, M., Kersters, K. and De Ley, J. 1992. Transfer of several phytopathogenic *Pseudomonas* species to *Acidovorax* as *Acidovorax avenae* subsp. *avenae* subsp. nov., comb. nov., *Acidovorax avenae* subsp. *citrulli*, *Acidovorax avenae* subsp. *cattleyae*, and *Acidovorax konjaci*. *Int. J. Syst. Evol. Microbiol.* 42:107-119.
- Zivanovic, M. and Walcott, R. R. 2016. Further characterization of genetically distinct groups of *Acidovorax citrulli* strains. *Phytopathology* 107:29-35.