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# Comparison of Atypical Bacteriophages That Infect Caulobacter Crescentus

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# COMPARISON OF ATYPICAL BACTERIOPHAGES THAT INFECT *CAULOBACTER CRESCENTUS*

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# ABSTRACT

Several Caulobacter bacterial genomes have been recently sequenced, and all of the genomes contained one or more clusters of genes with phage origins. This observation indicates that bacteriophages contribute to the Caulobacter gene pool, so in order to understand bacteria genomes we will need to understand phage genomes as well. As part of understanding the phage genomes, we want to isolate novel bacteriophages and study their genomics. This study resulted in the isolation of 12 new phages, including four that differ from the well-studied CbKlike phages. Two of these novel phages are Podoviruses with icosahedral heads and small tails, and one of these designated Lullwater, is similar to two previously isolated Caulobacter phages, Cd1 and Percy. They have a similar genome size around 45 kb and approximately 30 genes that are present in a conserved gene order. They also contain a T7like virus DNA polymerase, which classifies them as T7like Podoviruses. Based on these similarities, we concluded that Cd1, Lullwater and Percy comprise new Cd1like group in the T7like virus family.

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# CHAPTER 1

# INTRODUCTION

Caulobacter is a genus of oligotrophic bacteria found in fresh water and soil all over the world. They are rod-shaped, gram negative stalked bacteria. Bacteria in this genus divide asymmetrically, such that a stalked pre-divisional cell will divide into a flagellated cell called a swarmer cell, and a stalked cell. The swarmer cell is immature that must lose its flagellum and grow a stalk before it can replicate its chromosome and divide.

In 1999, the genus Caulobacter was split into two genera, Caulobacter and Brevundimonas, based on 16S rRNA gene sequences (Abraham et al. 1999). *C. crescentus*, *C. segnis*, *C. henricii*, and *C. fusiformis* remained in as Caulobacter genera while the other Caulobacter species clustered with the known species of Brevundimonas. Species in both genera have similar life cycles.

Several Caulobacter and Brevundimonas genomes have been sequenced and they all contain clusters of genes with a phage origin. Some of these genes are present in multiple genomes and others are unique to a particular genome (Scott and Ely 2016). Thus Caulobacter phages contribute to the bacterial gene pool, and to better understand Caulobacter genomics, we need to study phage genomics as well. More than 200 Caulobacter phages have been isolated and characterized, but prior to my study fewer

than 20 Caulobacter phage genomes had been sequenced. The best studied of these phages are giant DNA phages with a cylindrical head and a genome size of approximately 210 kilobase (kb) pairs exemplified by phage CbK (Agabian-Keshishian et al. 1970). Many of these phages are closely related, and share a high percentage of nucleotide identity. However, little was known about other types of Caulobacter bacteriophages. Therefore, we want to isolate novel phages that differ from Cbk to learn more about the atypical phages that infect Caulobacter and identify sources of DNA that correspond to the sequences observed in the bacterial genomes.

I have been collecting fresh water samples from a variety of locations, and have isolated a number of phages that infect Caulobacter crescentus strain CB15 or Brevundimonas sp. strain DS20. Many of the phages we have isolated seem to be giant phages around the size of CbK or even larger. Out of the 12 phages I have isolated to date, four Lullwater, Jessamine Road A, Jessamine Road B, and Smith were selected for further study. Lullwater, and Jessamine Road A are unique small phage with 40-50 kb genomes and few Caulobacter phages with genomes in this size range have been studied previously. Their small genome size was hypothesized early on based on the large plaque sizes the phages made in soft agar overlays. Jessamine road B and Smith were phages that infect Brevundimonas DS20. These two phages have genome size of 9 kb and are the first phages known to infect a Brevundimonas strain.

We sequenced the Lullwater, and Jessamine Road A genomes, and found that Lullwater is related to two previously isolated phages Cd1 and Percy. Jessamine Road A had a 45 kb genome that did not match any other phage genome present in the NCBI

database. Therefore, we chose to focus on Lullwater and compare the Lullwater genome to those of Cd1 and Percy.

# CHAPTER 2

## A GENOME COMPARISON OF T7LIKE PODOVIRUSES THAT INFECT *CAULOBACTER CRESCENTUS*

Numerous bacteriophages have been isolated that infect *Caulobacter crescentus*, a well-studied Alphaproteobacterium. A previous study (Johnson et al. 1977) showed that the *C. crescentus* phages have a highly specific host range and will not infect other bacterial genera. The most studied of these phages are a group of large DNA phages with a cylindrical head and a genome size of approximately 210 kilobase (kb) pairs exemplified by phage Cbk (Agabian-Keshishian et al. 1970). More recently Gill et al. (2012) isolated and sequenced the genomes of five additional CbKlike phages. Three members of this group, Magneto, Swift, and Karma, shared between 88 - 95% percent nucleotide identity across their whole genomes with only a few unique genes not present in the other genomes. In contrast, phages Rogue and Colossus, only shared 63% or 19% nucleotide identity, respectively, with the other CbKlike phage genomes. In addition, the Colossus genome was much larger (297 kb) than the other CbKlike genomes. Despite these highly divergent nucleotide sequences, the structural, DNA replication, and host lysis genes in these phage genomes share a common gene order. A subsequent paper by Ash et al. (2017) described the genome sequences of six additional CbKlike phages and compared them to the genomes of CbK and the five CbKlike phage genomes sequence

by Gill et al. (2012). They were able to identify more than 100 genes that were shared by all 12 phage genomes and the gene order of most of these genes was also conserved.

Another *C. crescentus* phage, Cr30, a T4-like transducing phage (Ely and Johnson 1977; Ely et al. 2015) has an icosahedral head with a rigid tail and is smaller than the CbKlike phages with a genome size of 155,997 bp. A phylogenetic analysis showed that its genome has diverged from that major groups of T4like phages that infect other bacterial genera but it has retained a conserved gene order that is present in most T4like phage genomes.

As part of a continuing survey of bacteriophages that infect Caulobacter, this paper describes three Caulobacter phages that are part of the *Podoviridae* family. Members of this group of phages have an icosahedral head with a short stubby tail. They have small genomes compared to those of the phages described above. One Caulobacter phage designated Lullwater that we isolated and characterized, proved to be a T7like member of the *Podoviridae* family. Genome sequence comparisons showed that the Lullwater genome was similar to those of two previously isolated Caulobacter phages, Cd1 and Percy. Cd1 was isolated in 1976 from a water treatment plant in Seattle (West et al. 1976). It has an icosahedral head that is 60 nm long and a short tail of about 10-12 nm. It has the ability to infect both the swarmer and stalked cell forms of Caulobacter. Percy is a similar phage that was isolated from a water sample obtained in College Station, Texas (Lerma et al. 2015). A comparison of these three phage genomes revealed that they form a unique branch of the T7likevirus phylogenetic tree.

Materials and methods

Isolation of bacteriophage from fresh water samples

The Lullwater bacteriophage was isolated from a water sample collected from the lake in the Lullwater Preserve in Atlanta, GA during late September, 2013 using SC1004, a streptomycin resistant mutant C. crescentus CB15 (Ely and Croft 1982), as a host. The 10 ml water sample was filtered through a 0.45 µm filter and then enriched for C. crescentus phage by adding 2.5 ml of 5X PYE broth (Johnson and Ely 1977), 100 µl of an overnight culture of SC1004, and 0.625 mg streptomycin sulfate. After overnight growth at 29 C, the phage enrichment culture was centrifuged twice at 8000 rpm for 10 minutes at 4 C, each time discarding the bacterial pellet. Then 1 ml of chloroform was added to lyse any remaining bacteria in solution. Next, the lysate was diluted one hundred-fold and 100 µl was added to 3.5 ml of PYE soft agar along with 100 µl of an overnight culture of SC1004. The mixture was immediately poured onto the surface of a PYE agar plate supplemented with 50 µg/ml streptomycin and then incubated overnight at 30 C. If plaques were observed, a sterile needle was used to stab a single plaque and transfer the phage particles into 1 ml of PYE broth. To further purify the phage the resulting phage suspension was diluted one hundred-fold and then 100 µl of the diluted suspension was mixed with 100 µl of host bacteria in 3.5 ml of melted soft agar and poured onto a PYE plate as before. After overnight incubation at 30 C, one of the resulting phage plaques was cut out from the soft agar overlay and placed in 1 ml of PYE broth. The resulting phage solution was diluted one hundred-fold the next day and again plated with host bacteria in a soft agar overlay. Since this second phage solution contained an entire plaque, several thousand plaques were present and a high titer lysate

was obtained by pipetting 5 ml of PYE broth onto the soft agar overlay. After allowing the phage to diffuse into the PYE broth overnight, the phage suspension was poured into a sterile test tube and the phage titer was determined using a series of serial dilutions.

Genome size determination

DNA agarose plugs were made by pipetting 1:1 ratio of phage lysate and melted 1% agarose into a 1 cc syringe. After cooling, the tip of the syringe was cut off and the solidified agar was sliced into 2 mM sections as it was extruded from the syringe. The resulting plugs were then incubated overnight at 50 C in 2 ml of a lysis buffer containing 1.9 ml of 1% Sarkosyl in 0.5 M EDTA and 0.1 ml of proteinase K (20 mg/ml). The next day the plugs were washed twice with 2 ml of TE buffer with 30µl of PMSF (17.4 mg/ml). Then the plugs were washed an additional three times with 2 ml of TE buffer. Once the washes were completed, the plugs were subjected to pulse field gel electrophoresis at 6 volts for 12 hours with a switch time of 1 second and compared to a lambda ladder (BioRad, Hercules, CA) to determine the size of the genome.

Phage particle concentration

The phage lysate was concentrated by ultracentrifugation at 25,000 rpm for 2.5 hours, and the resulting pellet was resuspended in 400 µl of 10 mM NaCl.

Electron Microscopy

A 1:1 mixture of host bacteria and concentrated phage lysate was incubated for half an hour at 30°C. Then, 30µl of 2% phosphotungstic acid was mixed with 30 µl of bacterial and phage mixture on a piece of Parafilm (Bemis NA, Neenah, WI) and then a coated copper grid was floated on top of the mixture.

DNA isolation for genome sequencing

Genomic DNA was isolated from the concentrated phage lysate using a Qiagen DNA isolation kit (Germantown, MD) according to the manufacturer's instructions.

Sequence Analysis and Assembly

DNA sequencing was performed by the University of Delaware using a PacBio sequencer. The resulting phage genome sequence was then assembled using the Hierarchical Genome Assembly Process 3 (HGAP3) (Chin et al. 2013) and then trimmed to remove repeated sequences at both ends of the genome. The terminal repeats that occur at the ends of the phage genome were located using Tablet to identify the region that contained twice the average number of reads, and the genome sequence was arranged so that the repeat region was at both ends of the linear genome. The resulting genome sequence was annotated using RAST (http://rast.nmpdr.org), and then visualized and edited in Artemis (Rutherford et al. 2000). The Cd1 and Percy genomes were downloaded from NCBI (https://www.ncbi.nlm.nih.gov). Multiple alignments of the three phage genomes were performed using Mauve (Darling et al. 2004). NCBI Cobalt (Papadopoulos and Agarwala 2007) was used to construct phylogenetic trees using predicted amino acid sequences downloaded from NCBI.

**Results**

The bacteriophage designated Lullwater was isolated from a water sample collected in Lullwater Park in Atlanta, Georgia during September 2013. Lullwater produces clear plaques that range in diameter from less than 1 mm to nearly 3.5 mm. It has a very narrow host range that includes CB15 but it does not infect other *Caulobacter*

species including *C. segnis*, CB4, APO7, and FWC20. Surprisingly, Lullwater does not infect other wild type *C. crescentus* strains including CB1, CB2, and CB13. The phage has an icosohedral head approximately 70 nm in length (Figure 2.1). This morphology indicates that Lullwater is part of the Podoviridae family.

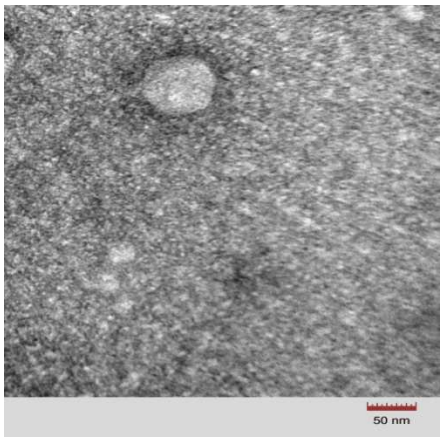


Figure 2.1. An electron micrograph of Lullwater phage particles.

Lullwater has a 46,531 bp double stranded DNA genome with a 54.5% GC content. It has 53 protein coding genes and one tRNA gene. The terminal repeats are 196 bp in length. To verify that the genome was correctly assembled, we performed a restriction enzyme digest and compared the results with the predicted band patterns. After digestion of the Lullwater genomic DNA with either *Pvu*II, pulsed field gel electrophoresis showed that there was a band at 26 kb and another one around 19-20 kb which matched the predicted bands indicating that the genome was complete and assembled correctly (Figure 2.2).

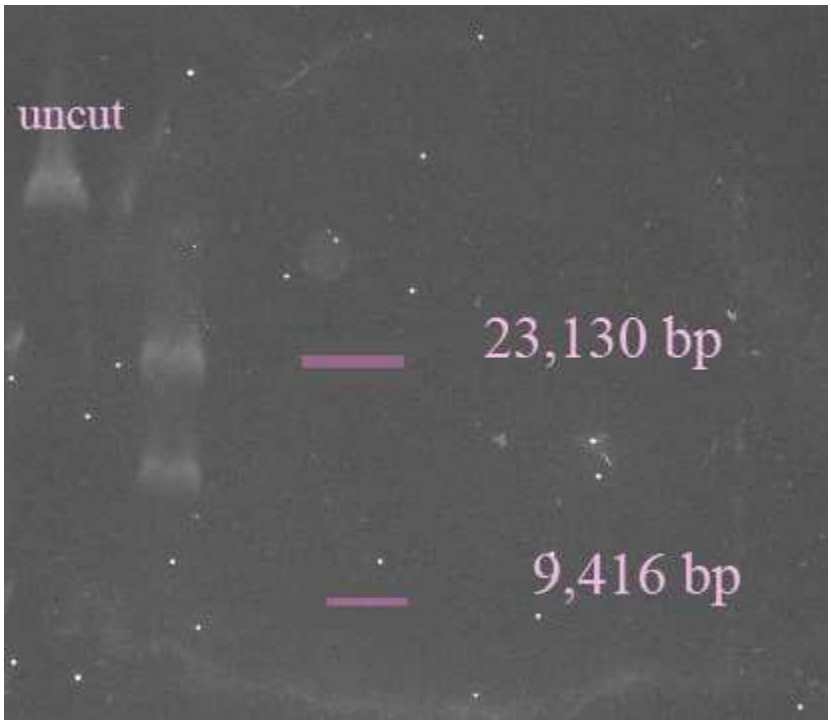


Figure 2.2. Agarose gel electrophoresis of a *Pvu*II restriction enzyme digest of Lullwater genomic DNA. Lane 1 uncut DNA; lane 2 *Pvu*II digested Lullwater genomic DNA.

The Lullwater genome arrangement includes early genes which code for all of the proteins needed to replicate the viral genome, then structural genes, then lysis genes (Figure 3). This genome organization is shared with the closely related to Caulobacter phages Percy (Lerma et al. 2015) and Cd1 (West et al. 1976). An alignment of the Lullwater, Percy, and Cd1 genomes showed that all three phages are homologous throughout the genome except for a region at the left side of the genome as depicted in Figure 4. The homologous genes in the three genomes share up to76% amino acid identity. The structural and DNA replication genes are the most highly conserved with the amino acid sequences of the DNA polymerases being similar to those of the T7like DNA polymerases. Thus these three phages can be considered T7like podoviruses. The remainder of the three genomes consists of genes coding for proteins with no known function (Table 2.1). One of these hypothetical proteins was 81% identical to a

hypothetical protein coded by the Percy genome, but the corresponding gene was not found in the Cd1 genome. Of the Lullwater unique genes, 17 were not found in any other phage genome in the NCBI database. Similarly, the Percy genome had 22 genes with no match in any other phage genome in the NCBI database and the Cd1 genome had 12.

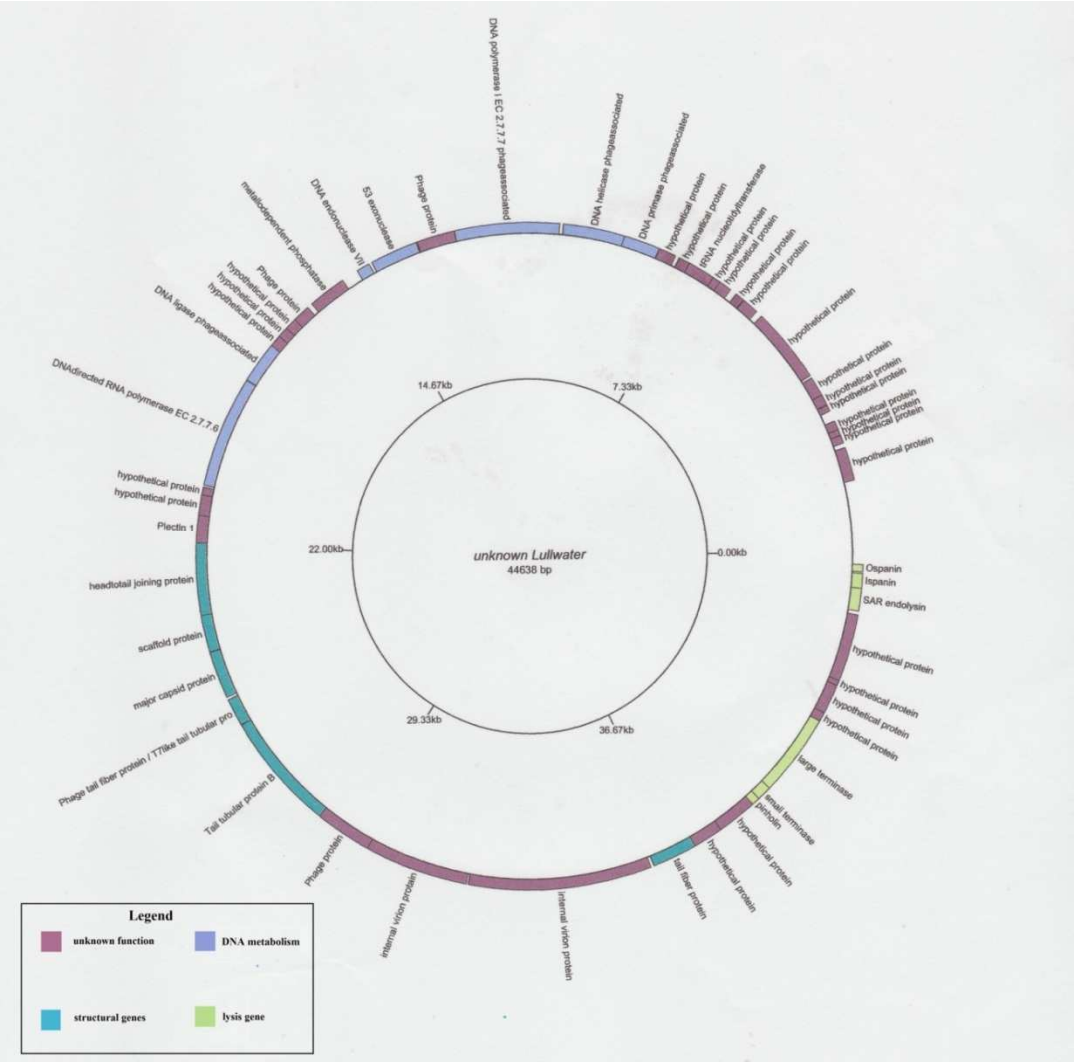


Figure 2.3. A circular representation of the Lullwater Genome. The image above shows the position of all annotated genes in Lullwater genome, and it is color coded based on functions of the genes. Position 0 indicates the position of the inverted repeats.

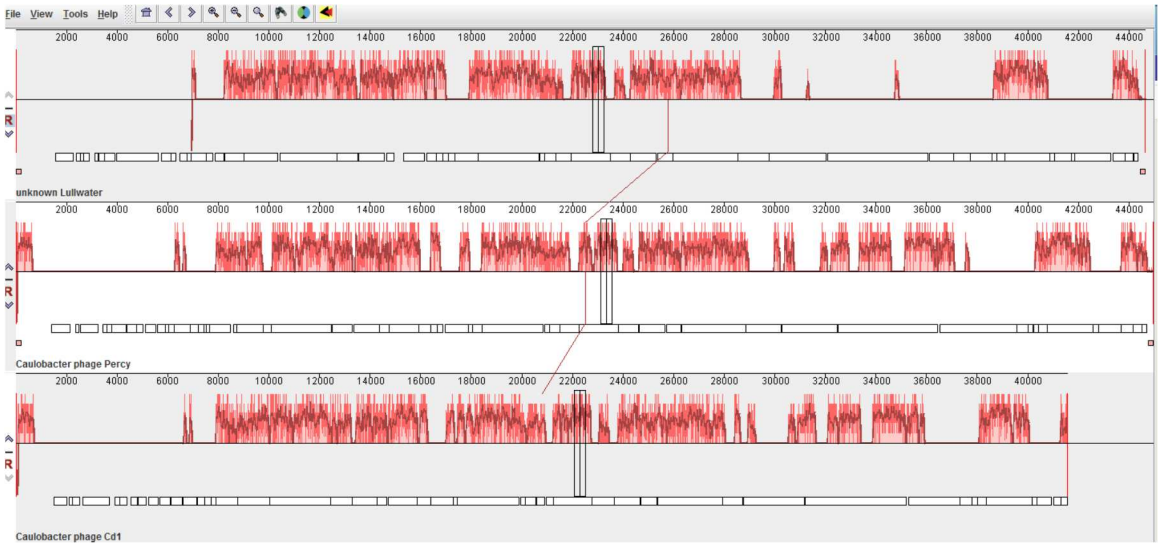


Figure 2.4 An alignment of Lullwater, Percy, and Cd1 genomes produced by Mauve (Darling et al. 2004). The dark line indicates the level of nucleotide identity. White blocks indicate the position and size of individual genes.

Table 2.1. Genes found in Lullwater, Percy, and Cd1

| Genes | Lullwater | Percy | Cd1 |
|---|---|---|---|
| Total | 53 | 55 | 47 |
| DNA replication | 9 | 9 | 7 |
| Structural | 7 | 6 | 6 |
| Lysis | 6 | 6 | 6 |
| Proteins of unknown function | 31 | 34 | 27 |
| Shared | 31 | 32 | 29 |
| Unique | 22 | 24 | 18 |

Hamdi et al. (2016) had previously demonstrated that the T7-like DNA polymerase genes were highly conserved and useful for showing the phylogenetic relationships of a large group of T7likeviruses. Therefore we constructed a phylogenetic tree using the amino acid sequences of representative T7likevirus DNA Polymerases, and showed that the amino acid sequences of the Lullwater, Cd1, and Percy enzymes cluster

together on a new branch of the tree (Figure 2.5). Similar phylogenetic trees were obtained when we compared the amino acid sequences of either the DNA-dependent RNA polymerases or the major capsid proteins of these phages (data not shown).

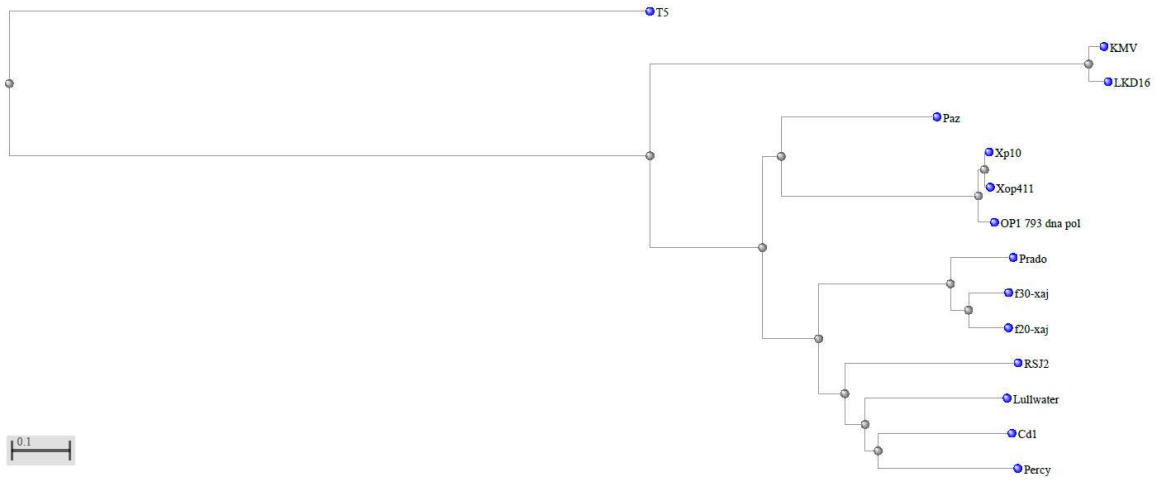


Figure 2.5. Phylogenetic tree based on the predicted amino acid sequence of the DNA polymerase of the T7like phages named in the figure.

**Discussion**

Based on the amino acid homology of conserved genes, Lullwater is most closely related to the Cd1 and Percy Caulobacter phages, and together the three phages form a new branch of the T7likevirus phylogenetic tree (Figure 2.5). However, unlike many of the other branches of the T7likevirus tree, no close relatives of any of these three phages have been identified. This situation is likely to be remedied as more Caulobacter phages are isolated and characterized in our laboratory and elsewhere. In addition, it will be interesting to see if this branch of the T7likevirus tree contains phage that infect other hosts or whether it is restricted to phage that infect *Caulobacter crescentus*.

Unlike other Caulobacter phages that have been characterized, Lullwater is only able to infect our CB15 strain. It does not infect NA1000, which is another version of

CB15. The two strains differ primarily by the absence of a 26 kb segment that is missing in the CB15 genome and present in the NA1000 genome. Thus, the presence of a gene in that region could be preventing Lullwater from infection other *C. crescentus* wild type strains. The 26 kb region contains a number of genes that are involved in glycosylation so it is possible that the phage receptor is modified in these strains, preventing the phage from infecting the bacteria

Despite the absence of genes that code for proteins with amino acid identities greater than 80%, the gene order is conserved across the Lullwater, Cd1 and Percy genomes. This phenomenon is not surprising since, the gene order is generally conserved across nearly all of the knownT7likeviruses with only a few phages that deviate slightly from the conserved organization (Ahern et al. 2014, deLeeuw et al. 2017, Hamdi et al. 2016, Lavigne et al. 2003, Scholl et al. 2004).

In regions of the Lullwater genome that did not align with the Cd1 and Percy genomes (Figure 2.4), there were a number of genes that code for unique hypothetical proteins with most of these genes found at the beginning of the Lullwater genome. Likewise, most of the genes coding for unique proteins were located at the beginning of the Cd1 and Percy genomes as well. This gene arrangement is similar to those found in other T7likeviruses. For example, many of the genes that code for hypothetical proteins in the KMV genome also were found towards the beginning of the genome. However, wherever closely related phage genomes have been identified, these hypothetical protein genes were shared among the close relatives. This observation indicates that the beginning of the T7likevirus genomes is much more variable than the remainder of the

genome suggesting that the functions of these genes may be involved with host range or other aspects of phage biology where genetic variation may be beneficial.

Two genes in the Lullwater genome code for hypothetical proteins that are 34% and 60% identical to proteins produced by Cr30, a T4like transducing phage that also infects CB15 (Ely and Johnson 1977, Ely et al. 2015). The presence of Lullwater genes that are homologous to Cr30 genes indicates that horizontal transfer events may have occurred when Cr30 and Lullwater ancestors co-infected the same host bacterium. One of these Cr30-like proteins is also found in Cd1, but not in Percy, suggesting that a horizontal gene transfer event may have occurred between a Cr30 ancestor and a common ancestor of Lullwater and Cd1. It is also possible that a horizontal gene transfer event occurred between the Cr30 ancestor and a common ancestor of Lullwater, Cd1 and Percy, and then Percy subsequently lost the gene. A similar example of possible horizontal gene transfer has been observed in a CbKlike phage where recombination with a CR30-like phage appears to have caused a genome rearrangement (Ash et al. 2017).

In summary, we have described a new group of T7like phages that infect *Caulobacter crescentus*. As we continue to isolate and characterize novel phages that infect various *Caulobacter* isolates, it will be interesting to determine if all of the T7like isolates are part of this groups or whether they can be found in other branches of the T7like phylogenetic tree.

# CHAPTER 3

# CONCLUSIONS

During the past two years, the Ely laboratory, in collaboration with the bacteriology laboratory course students, has isolated more than 100 new bacteriophages that infect Caulobacter. More than 80% of these phages are CbKlike phages with elongated heads and flexible tails. All of the Cbklike phages characterized to date have related genomes (Ash et al. 2017). However, as we sequence additional CbKlike genomes, we are identifying divergent branches of the CbKlike phylogenetic tree (Wilson et al., in preparation).

The non CbKlike phages have smaller genomes that are mostly in the 40 to 50 kb size range. So far, three of these phage genomes have been sequenced and four additional 40 to 50 kb phage genomes have been sequenced in other laboratories (Lerma et al. 2015, Sloan et al. 2015, Vara et al. 2015). In contrast to the Cbklike phage genomes, most of these smaller phage genomes are unrelated to each other and are not related to any other phage in the GenBank database. The exception is Lullwater whose genome is distantly related to the Percy and Cd1 genomes.

Lullwater is a small Podovirus that infects CB15. Its closest relatives are Caulobacter phages Cd1 and Percy. These three phages form a new branch of phages in the T7like phage family. Unlike other phages that infect Caulobacter, Lullwater does not

infect other *C. crescentus* wild type isolates, which makes it unique. For example, RW, another small phage with a genome size similar to Lullwater, has the ability to infect all the other *C. crescentus* species along with CB15. The Lullwater host range also differs from that of CD1 and possibly that of Percy since CD1 can infect CB13 and the host range of Percy has not been determined. Further studies will be needed to determine why the host range of Lullwater is so restricted.

This study is just the beginning of a long term study of the small phages that infect *Caulobacter* and *Brevundimonas*. The seven genomes sequenced to date resulted in the Lullwater cluster of three distantly-related genomes and four genomes that are each unrelated to any known phage. We have several isolated at least 10 additional small phages whose genomes will be sequenced. Based on these data, we believe that we have not found all the types of small phages that infect *Caulobacter* and *Brevundimonas*. Therefore, the Ely lab plans to continue isolating and sequencing small phage genomes until we find multiple isolates of each type of small phage so that we can have a more comprehensive understanding of the pool of genes that these small phages can contribute to the *Caulobacter* and *Brevundimonas* genomes.

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