First in Phlight: Characterization of Two Novel Phages, Iridoclysis and Nidhogg, Isolated in North Carolina

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Introduction

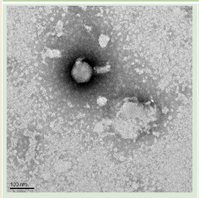
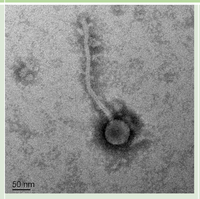
- The world's dangerous dependence on antibiotics has led to renewed interest in the potential use of mycobacteriophages either prophylactically or therapeutically in the treatment of tuberculosis[1].
- To contribute to the genomic characterization of mycobacteriophages, we used *Mycobacterium smegmatis* as a host to isolate two novel bacteriophages, Iridoclysis and Nidhogg, by enrichment in Fall 2015.
- Both bacteriophages were annotated and characterized by bioinformatics and laboratory experiments.

Nidhogg Characteristics:

- Found in Raleigh, NC, in a grassy, semi-fertilized area, frequently flooded with rainwater.
- A subcluster C1 phage with 156342 bp.
- 65% GC content displaying a *Myoviridae* morphotype.
- 35 tRNAs identified within the genome.

Iridoclysis Characteristics:

- Found on campus of Durham Technical Community College in a flower bed.
- A subcluster B1 phage with 68587 bp.
- 66.4 % GC content displaying a *Siphoviridae* morphotype.

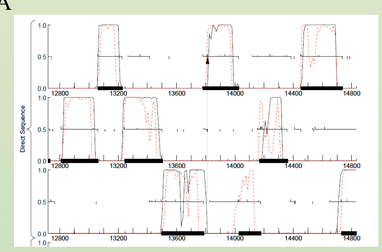
Nidhogg	Iridoclysis
 Subcluster C1 phage *Myoviridae* morphotype Average tail length: 75nm	 Subcluster B1 phage *Siphoviridae* morphotype Average tail length: 225nm

Genome Annotation

- DNA Master, PhagesDB, NCBI BLAST, Phamerator, Starterator and HHpred software programs were used to identify genes and gene functions.
- Web-based programs, Aragorn and tRNAscan-SE, were used to find tRNA genes and identify their correct ends.

Annotation of an Additional Gene Not Called by DNA Master to Nidhogg's Genome

A



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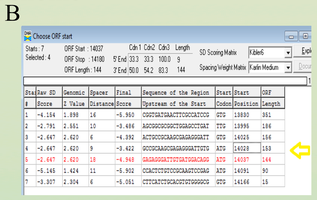

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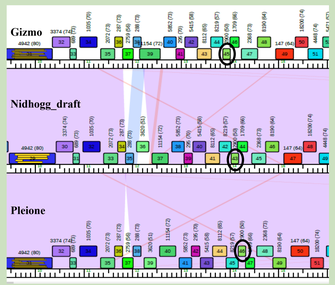
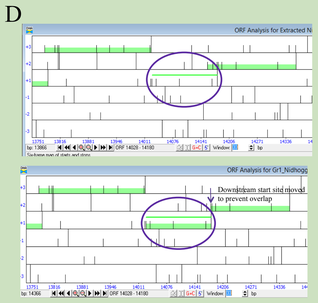


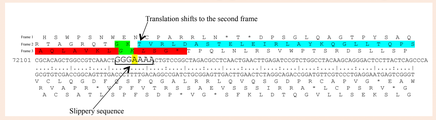
D

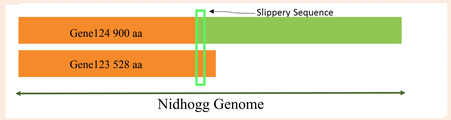


A. Gene prediction program, Genemark, shows coding potential between 14020-14200 bp.
B. The best genomic Z score comparing ribosome binding sites (Z = 2.6) gave further confidence for annotation of a 153 bp gene with start site at 14028 bp. Amino acid alignment using the protein BLAST program, BLASTp, showed many 1:1 protein alignments starting at 14028bp (not shown).
C. Genome exploration tool, Phamerator showing presence of an annotated gene in this genomic area in highly similar-sequence phages, Gizmo and Pleione.
D. DNA Master image showing before and addition of the gene.

Annotation of the Tail Assembly Chaperone Translational Frameshift in Nidhogg

Nidhogg has a -1 Programmed Translational Frameshift

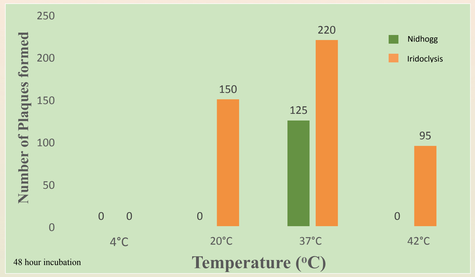




- In many bacteriophages, a programmed translational frameshift makes two proteins with overlapping amino acid (aa) sequences essential for tail assembly.
- Frameshifts occur when there is a slippery sequence in the mRNA where the ribosome can occasionally change reading frame and translate an alternate protein
- In Nidhogg, the ribosome enters the "slippery" sequence GGGAAAA in the 3rd reading frame (red) of gp123.
- The ribosome either continues in the third reading frame producing a 528 aa protein or shifts back one base pair on the mRNA into the second reading frame and continues to the end producing a 900 aa protein.
- The frameshift coordinate was identified at 72127 bp where the A (yellow) is counted twice in translation.

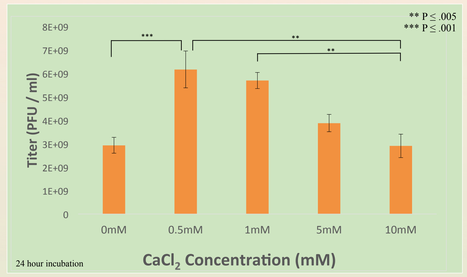
Results from Experimental Characterization

Effect of Temperature on Plaque Formation



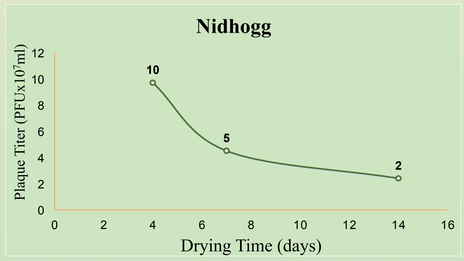
- Iridoclysis demonstrated ability to grow over a range of temperatures (20°C, 37°C, 42°C) with an optimal temperature of 37°C.
- Nidhogg showed a limited tolerance to the range of temperatures tested, only optimally producing plaques at 37°C.

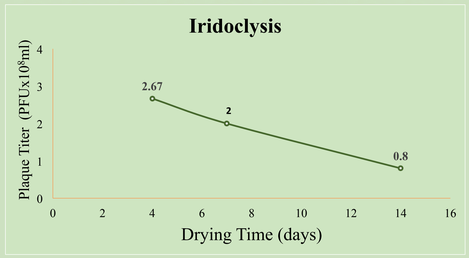
Iridoclysis Plaque Formation is Sensitive to Calcium Concentration



- Iridoclysis was grown over a range of top agar calcium concentrations (0 – 10 mM; n = 4); error bars represent mean ± SD, *P* values calculated by one-way ANOVA.
- The optimal calcium concentration was found to be 0.5 mM.

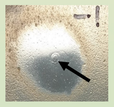
Effect of Drying on Phage Stability



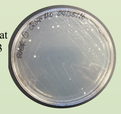


- Dried out plaques from both Iridoclysis and Nidhogg were reconstituted in 15 ml after a range of drying times (4 – 14 days), 10 ml was mixed with *M. smegmatis* and the phage titer was calculated after 24 - 48 hours of incubation.
- A decrease in phage stability proportional to the drying time was observed.
- Notably, both phages were still producing plaques after two weeks of drying, demonstrating resilience to less than ideal conditions.

Nidhogg is capable of making lysogens



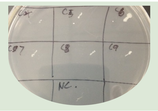
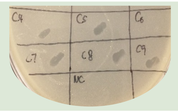
A "mesa" formed from the spot test at high titer in 1 of 3 experiments .



Cells from the mesa were streaked onto new plates. After 3 days incubation at 37°C, Bacterial colonies were observed which contained potential lysogens.

- When 10-fold dilutions of Nidhogg were spotted on a lawn of *M. smegmatis,* a turbid circle in the center of the plaque – a "mesa" – was observed, suggesting the presence of lysogens.
- Iridoclysis did not produce lysogens in the three experimental trials performed.

Patch test and spontaneous plaque production verified the presence of lysogens

Agar Plate	Agar Plate with *M. smegmatis* lawn
	

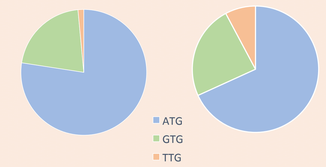
Lysogens produced plaques on *M. smegmatis* lawn

- Potential lysogens from C7-C9 (above) were streaked for purification.
- A liquid culture was then inoculated with one purified colony to produce a lysogen stock.
- To verify the lysogen, filtered supernatant from the lysogen stock was tested for spontaneous plaque production by performing a spot titer on host bacteria. The lysogen titer was **1.8×10^6 PFU / ml.**
- Further tests are necessary to determine stability of lysogens from Nidhogg.

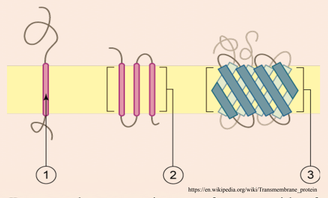
Bioinformatics Experiments

Translation Initiation (Start) Codon Preference

- Mycobacteriophage genomes use ATG, GTG or TTG as start codons.
- We calculated that Nidhogg has a preference for ATG as a start codon: 78.2% of genes used ATG; 21.3% GTG and 1.5% used TTG.
- Iridoclysis also has a preference for ATG: 68.2% of genes used ATG, 24.0% used GTG, and 7.8% used TTG.



Potential Transmembrane Protein Identification in Nidhogg and Iridoclysis



Transmembrane proteins span from one side of the membrane to the other at least once.

No.	N terminal	Transmembrane region	C terminal	Type	Length
1	34	IAKAVAAFVAALGGMAAVLLADA	56	PRIMARY	23
2	65	NIIAILGSLVAAGVVAGVVFKVP	87	PRIMARY	23

- Identification of the two transmembrane helices in Holin, a protein characterized by presence of transmembrane domains.
- Holin aids in release of phage particles from an infected host cell.
- Holins are extremely diverse and have not been identified for all phages[2] including Iridoclysis.

- The functions of many bacteriophage genes remain unknown. To identify proteins containing transmembrane domains, batch amino acid sequences were entered into the online secondary structure prediction tool, SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/).
- We identified 18 potential transmembrane proteins in Nidhogg and 10 in Iridoclysis.
- Known transmembrane proteins identified included the Tape Measure Protein and Lysin B in Iridoclysis and Holin in Nidhogg.
- Identifying transmembrane proteins may help shed light on bacteriophage proteins of unknown function and potentially identify Holin proteins or additional proteins involved in host cell lysis.

Conclusions

Nidhogg

- Nidhogg is a 156 kb subcluster C1 phage with 65 % GC content.
- Annotation was manually refined for a total of 272 open reading frames - 266 genes are transcribed in forward orientation and 6 genes in reverse orientation.
- Consistent with other C1 phages, a -1 programmed translational frameshift was annotated in the tail assembly chaperone genes and 35 tRNA genes were annotated.
- Growth of Nidhogg is temperature sensitive with an optimal temperature of 37 °C. Growth was severely disrupted at the other temperatures tested.
- Nidhogg is capable of producing lysogens and further work is required to determine the immunity status and whether the lysogens produced are stable.
- Stability of Nidhogg and Iridoclysis decreases with drying time, but plaques could still be produced for up to two weeks.

Iridoclysis

- Iridoclysis is a 69 kb subcluster B1 phage with 66.4 % GC content.
- Annotation was manually refined for a total of 101 open reading frames - 49 genes are transcribed in forward orientation and 52 in reverse orientation.
- Consistent with other B1 phages, no programmed translational frameshift was annotated and no tRNA genes were identified.
- Iridoclysis is less temperature sensitive then Nidhogg growing at all tested temperatures except 4 °C. The optimal temperature was 37 °C. Lack of growth at 4 °C is explained because *M. smegmatis* did not grow at 4 °C.
- Iridoclysis grew optimally when 0.5 mM $CaCl_2$ was added to the top agar. The phage titer decreased with increasing calcium concentration.

Acknowledgments and References

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- Sean Turner and James Pratt for discovery of Nidhogg and Iridoclysis , respectively
- UNC-CH microscopy core for electron microscopy
- Melinda Grosser for teaching assistance during the Phage Discovery semester

1. Hatfull, PLOS Pathogens 2014, 10
2. Catalão, J. Bacteriol 2011, 193