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Identifying the Cytotoxic Effects of Mycobacteriophage Genes

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I. Abstract

A bacteriophage is a virus that infects and reproduces in bacteria. During productive infections – those that result in construction and release of infectious phage particles – key host cell metabolic processes are modified by the infecting phage and redirected toward making new phage particles. Protein-protein interactions are likely involved in this process. In this work, gene 80 of mycobacteriophage Vix, a gene cytotoxic to host strain *Mycobacterium smegmatis*, was studied. Our hypothesis was that an interaction between the Vix80 gene product and a host cell protein caused growth inhibition. The Vix80 protein shares 68% amino acid identity with the product of gene 77 of mycobacteriophage L5. The L5_77 protein has been previously shown to exhibit cytotoxic properties, and interacts with MSMEG_3532, a L-serine dehydratase. The Vix80 and MSMEG_3532 proteins were expressed in *Escherichia coli* and purified, but attempts to show a physical interaction *in vitro* have not succeeded. In addition, no interaction between Vix80 and MSMEG_3532 was observed through two-hybrid analysis. HHMI-Pred analysis found that Vix80 contains a conserved domain of unknown function (DUF) near the N-terminus. The Vix80 gene was dissected, and the N-terminal 66 residues, encompassing the entire DUF, was found to be cytotoxic to *M. smegmatis*. DUF was found to be homologous to a region of three *M.smegmatis* ORFs, two of which are related by alternate initiation points of the same sequence. Efforts to test the interaction of Vix80 and the VIX80 DUF with themselves and the three host proteins are described. Identifying the relevant phage and host gene products and understanding how phage exploit their host's weaknesses could lead to new therapeutic options for many bacterial illnesses.

II. Background

A. Host organism statistics

- > 100 *Mycobacterium* species reported; includes a pathogen that causes tuberculosis, affecting 1/3 of world population
- Multiple species and strains are receptive to infection by mycobacteriophages
- Full genome sequences of mycobacteriophage reveal many novel genes (1), including several that are cytotoxic to *M. smegmatis mc²155* (2)

B. Mycobacteriophage Vix

- Isolated in Fall 2009, Hope College Phage Genomics Research lab course
- 50,963 bp, 64% GC, 82 protein-encoding genes, 3 tRNA genes
- Member of the A3 subcluster of mycobacteriophages (3)
- Related phages Che12, Bxz2, L5 have been shown to infect *Mycobacterium tuberculosis* (4,5)

C. Mycobacteriophage Vix80 gene product

- Vix80 gene product exhibits high amino acid identity to L5 gp77 (68%) and related mycobacteriophage proteins (Figure 1)
- Vix80 gene product contains a conserved but functionally undefined N-terminal domain (DUF2786)
- L5 gp77 is cytotoxic to *M. smegmatis* and interacts with host cell protein MSMEG_3532, an L-serine dehydratase (2,6)

III. Results

A. Vix 80 and L5_77 Protein Similarities

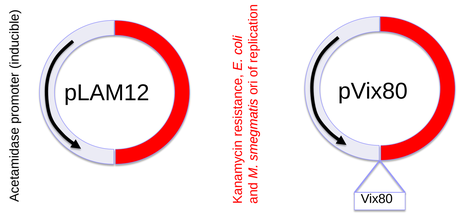
Figure 1- Protein Alignment Results Between L5_77 and Vix_80

```
Vix_80 1    MDGKTAKMQKQVAKLLRHAEDVVGTPEEAVFMAKAFELIAKYGLDMASIQADKQGLDTSDMPDAIKWSARIDGKYTAQQVLLLHGLVVAL  90
            +DGKT KMQ +VAKLLR AEDV GTPEEAVF AKAFEL+AKYGL+MA ++A KQGLDT+D+PDAI+W   + GKY A Q LLLHG+  AL
L5_77  2    IDGKTKKMQDKVAKLLRQAEDVAGTPEEAVFQAKAFELMAKYGLEMAQVEASKQGLDTTDLPDAIQWVTYVTGKYVAAQALLLHGMAQAL  91

Vix_80 91   HCKAVMTHEVSSGTRYPVLHIFGVPRHIERVQFLWEILRPQMLRLVENVRPAEGFTPRYTYDYRTGEYRQKKTSGQLKSYRRAWIAGFAQ  180
            HCK V    ++ G R   ++++GVPRHIER+QFLW I++PQM+RLVENVRP + F PRY YDY TGEY+ K T+GQLKSYRR+WIAGFAQ
L5_77  92   HCKTVYA-SLTGGQR---IYVYGVPRHIERLQFLWSIMQPQMMRLVENVRPEQAFEPRYKYDYNTGEYKPKSTAGQLKSYRRSWIAGFAQ  177

Vix_80 181  TVSERVKAQEDKVLESAESGALVLYRGDKERAALALREAFPRTRRARGRTRFDSNGYAHGQRDGRNAAFARALA  254
             V ER+ A+E+K +ESA  GALVLYRGDKERAALALR+A PRT+  + RTR+D +GYAHGQRDGRNAA  ALA
L5_77  178  AVKERLTAEENKAVESAGGGALVLYRGDKERAALALRQAHPRTKNVKPRTRYDLSGYAHGQRDGRNAAMNHALA  251
```

Protein BLAST between mycobacteriophage L5 gp77 and mycobacteriophage Vix gp80. The BLAST results show alignment over 99% of both gene products and 68% amino acid identity. Amino acids shown between aligned mycobacteriophage protein sequences represent conserved residues. Residues highlighted in orange box near N-terminus of Vix gp80 and L5 gp77 match the functionally undefined conserved protein domain DUF2786.

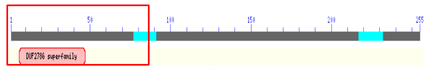
B. Cytotoxicity of Vix80

Figure 2- Vix80 is cytotoxic to *M. smegmatis*



Parent vector and recombinant pVix80 plasmid used to control Vix80 gene expression and test for cytotoxicity in *M. smegmatis* cells. Vix80 coding sequence was amplified by PCR from Vix genomic DNA and ligated adjacent to the acetamidase promoter in expression vector pLAM12. Recombinant pLAM12 plasmids were constructed using standard recombinant DNA protocols and used to transform *M. smegmatis* mc2155 cells via electroporation. Larger genomic sections were cloned from restriction enzyme digests of genomic DNA. Short genomic regions (single to several genes) were cloned following PCR amplification.

Figure 4. Vix80 gene conserved domain location



Protein residue map of Vix80 with the conserved domain. The red box indicates what section of the gene is makes up the N-terminal region used in later cytotoxicity tests.

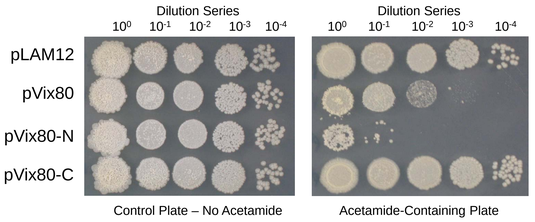


Figure 5. Cytotoxicity testing of Vix80 N-terminus and C-terminus
Overnight cultures of transformed M. smegmatis (MC²155) were diluted and spotted (2.5 µl) onto control (no acetamide) and test (with acetamide) media. Plates were incubated 3-4 days at 37°C.

C. 2-hybrid Interaction Trap

- Tests for physical interactions between proteins by recapitulating a functional transcription factor with both DNA binding and activation domain. The two domains are separated by molecular methods onto two different plasmids. A chimera between each of the two putative interacting proteins (such as Vix80 and MSMEG_3532) are created, attaching one to the bait, and one to the prey.
- A DNA binding domain (pEG202) binds to a regulatory DNA sequence.
- An activation domain (pJG4-5) activates RNA polymerase.
- If the DNA binding domain and the activation domain are brought together through protein interaction, they can activate a downstream reporter gene.

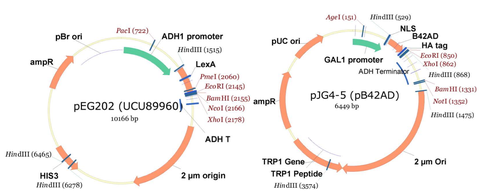
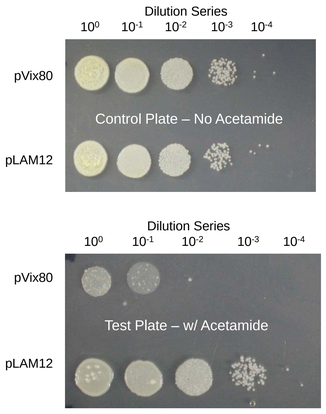


Figure 6 Bait and Prey plasmids used in this study

Figure 3- Vix80 cytotoxicity plate test



Cultures were grown at 37°C in 7H9 medium containing OADC supplement (10%), Tween 80 (0.5%) and kanamycin antibiotic (25 µg/mL). Saturated overnight cultures were serially diluted before spotting (2.5 µL) onto 7H10 agar plates supplemented as indicated above and either lacking acetamide (control condition) or containing 0.1% final concentration of acetamide (to induce expression). Spotted cultures were incubated for 3-4 days at 37°C and examined for growth. Equivalency in the numbers of cells spotted was assessed by comparing growth across all strains on the control medium (no acetamide). Cytotoxicity was assessed by comparing quality of growth for each strain under control (no acetamide) and test (acetamide supplemented) conditions.

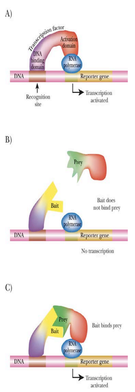
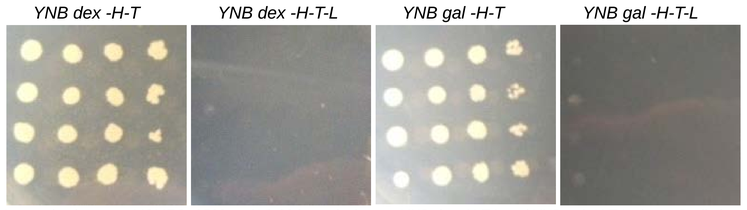


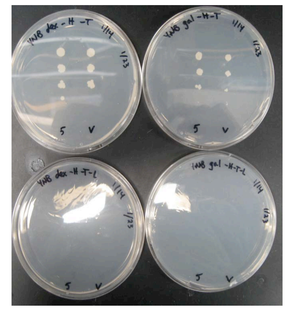
Figure 7- The 2-hybrid interaction trap
From *Molecular Biology,* 2nd ed. Clark and Pazdernik.

Figure 8- 2-hybrid interaction trap shows no interaction between MSMEG_3532 and Vix80



Interaction between MSMEG_3532 and Vix80 is equivalent on YNB dex -H-T-L and YNB gal -H-T-L plates. Preliminary results suggest that the Vix80 gene may be reacting with itself to form a homodimer, rather than reacting with MSMEG_3532.

Figure 9- 2-hybrid interaction trap does not show interaction between Vix80 and MSMEG_1191
To identify specific sequences of *M. smegmatis* that encode proteins that may interact with Vix80, three sequences were found with homology to the DUF region of Vix80: msmeg_0222, msmei_1191, and msmeg_1225. These genes were identified as encoding histone-like proteins. Sequence msmei_1191 was ligated into yeast vector pEG202 and tested for interaction in a two-hybrid system with pJG4-5-Vix80. While no interaction was observed, without a positive control, results still need verification.



IV. Conclusions

- Vix gp80, a homologue of the cytotoxic gp77 of mycobacteriophage L5, is cytotoxic to *M. smegmatis*
- Vix80 does not interact with *M. smegmatis* 3532, thought to be the interacting protein with L77
- Vix80 contains a domain of unknown function (DUF) near the N-terminus. A region of 66 residues near the N-terminal was dissected, encompassing the entire DUF, and was found to be cytotoxic to *M. smegmatis*
- Three Vix80 does not interact with *M. smegmatis* 3532 genes were identified that contain a DUF homology domain
- Vix80 does not interact with DUF homology containing protein *M. smegmatis* 1191

V. Questions & Future Plans

Questions-

- How does the conserved but functionally undefined N-terminal domain and the C-terminal domain of Vix gp80 relate to Vix80 cytotoxicity?
- Is the Vix80 phage gene essential for Vix phage infection and reproduction in *M. smegmatis*?
- Does Vix gp80 physically interact with *M. smegmatis* proteins 0222 or 1225?

Future Plans-

- Investigate whether the Vix80 DUF is needed for protein-protein interaction
- Delete the Vix80 gene in the Vix phage genome and test for the ability of phage Vix to infect and reproduce in *M. smegmatis*
- Determine if Vix80 interacts with *M. smegmatis* proteins 0222 or 1225 by two-hybrid analysis
- A positive control for the 2-hybrid system is being constructed using the genes RAF and RAS

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Acknowledgements

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